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JOURNAL OF THE AMERICAN PHARMACEUTICAL ASSOCIATION

JUSTIN L. POWERS, EDITOR, WASHINGTON, D. C.

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The Determination of Estrone, Equilin, and Equilenin by Infrared Spectrophotometry*

By JONAS CAROL, J. C. MOLITOR, and EDWARD O. HAENNI†

A method is described for the determination of estrone, equilin, and equilenin in mixtures by infrared spectrophotometry. Optical density measurements are made at a selected absorption peak for each component, and from these data the concentration of each ketosteroid is determined by a graphical approximation method. The results of analysis of a series of control samples are reported.

THE PRINCIPAL commercial source of estrogens is the urine of gravid mares which contains a rather complex mixture of these substances. The usual method of estimating the potency of material from this source, based on its physiological activity as compared to that of pure estrone, does not permit the determination of the identity or relative proportions of the various estrogens in the mixture. It seemed desirable, therefore, to develop a procedure which would give a more complete picture of the composition of these products.

The ketonic fraction of these hormones is generally supposed to be composed chiefly of estrone along with small and varying amounts of equilin and equilenin (1, 2). The presence of hippulin reported by Girard and his co-workers (3) has never been substantiated. In a previous paper we (4) described a method using Girard Reagent T (5) for the estimation of total ketosteroids, as estrone, in oil solutions of natural estrogens. The purpose of the present work was to devise a procedure for the determination of the components of this ketosteroids group.

A number of methods are found in the literature for the separation of these compounds. Sandulesco and his co-workers (6) precipitated equilenin from estrone and equilin as the insoluble picrate. Veitch and Milone (7) separated the dinitrophenylhydrazones of estrone and equilenin by chromatographic absorption. Serini and Logemann (8) reduced estrone and equilin to α-estradiol and dihydroequilin with sodium in alcoholic solution and then separated α-estradiol as the insoluble digitonide or double urea compound. None of these methods appeared to be suitable for the quantitative determination of the small amounts of ketosteroids (usually 5–10

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mg.) obtained in the analysis of commercial estrogenic products.

The ultraviolet absorption of estrone has been used for its quantitative determination (9, 10). Its ultraviolet spectrum is identical with that of equilin having a peak in the region of 280 mμ and no absorption beyond 300 mμ. The ultraviolet absorption spectrum of equilenin, due to the benzenoid B-ring, is much more complex showing peaks at 270, 280, 292, 328, and 348 mμ. Consequently, equilenin could be determined by ultraviolet spectrophotometry in the presence of estrone and equilin, but the amounts of estrone and equilin could not be estimated.

Furchgott, Rosenkrantz, and Shorr (11) have measured the infrared absorption spectra of estrone, equilin, and equilenin using solid films deposited on rock salt plates by the evaporation of pyridine solutions of the ketosteroids. Each of these spectra has at least one region in which it differs from the others. Dobriner and his coworkers (12) have recently reported a procedure for the identification of urinary ketosteroids by infrared analysis.

Quantitative infrared analysis of multicomponent mixtures is now widely used. A typical example is the method for the analysis of the isomers of hexachlorocyclohexane developed by Daasch (13). In this procedure optical density measurements are made at selected wave lengths for each component using a carbon disulfide solution of the sample. At each of these wave lengths one component has strong absorption while the others have weak absorption. The concentration of each component is determined by a graphical method using working curves prepared from the pure isomers. A similar technique was applied to the present problem.

Carbon tetrachloride and carbon disulfide are the best solvents for infrared studies since they are relatively transparent, in appreciable thickness, throughout the infrared spectrum (CCl_4, 2–7 μ; CS_2, 7–15 μ). Since estrone, equilin, and equilenin are practically insoluble in these solvents, it was necessary to convert them to their esters to effect solution.

The benzenesulfonyl ester was used in this investigation because it had the following distinct advantages over others:

1. It was easily prepared quantitatively by the reaction of the ketosteroid with benzenesulfonyl chloride in pyridine.
2. The benzenesulfonyl esters showed the greatest differences in absorption spectra of those examined.
3. The benzenesulfonyl ester does not involve the introduction of a carbonyl group as in the case of the acetyl or benzoyl ester. While no use was made of this property in the present work, it would be possible to determine small amounts of ketosteroids (as benzenesulfonyl ester) using the very specific and intense C=O absorption in the 5.75 μ region of the spectrum.

EXPERIMENTAL

The instrument used was a Perkin-Elmer Model 12B recording infrared spectrometer equipped with a sodium chloride prism and a lithium fluoride shutter. It was fitted with an automatic slit-control mechanism designed by Plyler, Stair, and Humphreys (14). This device continuously opens the slit as the spectrum is scanned from short to long wave lengths and produces an almost constant energy level from 3.5–15 μ. The wavelength calibration was made using the water vapor and ammonia spectra.

The absorption cells used were similar to a type designed by Tufts and Kimball (15). Details of construction are shown in Fig. 1. Gaskets and spacers were made of polyethylene which is unaffected by carbon disulfide. After the cells were assembled they were slowly heated in an oven at 105°. The polyethylene is thermoplastic and flows slightly forming a perfect seal. For quantitative measurements, cells with 0.5-mm. spacers were used. These cells hold about 0.2-ml. solution. No attempt was made to have pairs of cells match exactly, but the difference in optical density between the sample cell and blank cell was carefully determined at the various wave lengths used and checked frequently.

Estrone Benzenesulfonate.—Fifty milligrams of estrone (m. p. 259.0°, Sp. R. + 158° [dioxane]) was dissolved in 5 ml. of dry pyridine in a small glass-stoppered flask. One-half milliliter of benzenesulfonyl chloride was added, the flask was tightly stoppered and allowed to remain overnight at room temperature. The reaction mixture was diluted with 50 ml. water, transferred to a 125-ml. separatory funnel, and extracted with three successive 25-ml. portions of chloroform. The combined chloroform extract was evaporated to dryness on a steam bath in a current of air. The residue was recrystallized three times from 90% methanol. Yield 55 mg., white, crystalline powder, m. p. 153–154.2° (corr.).

Equilin Benzenesulfonate.—Prepared as above using 50 mg. equilin (m. p. 239°, Sp. R. + 304° [dioxane]). Yield 62 mg. white, crystalline powder m. p. 138.4–139.6° (corr.).

Equilenin Benzenesulfonate.—Prepared as above using 50 mg. equilenin (m. p. 157–158.5°, Sp. R. + 88° [dioxane]). Yield 65 mg. white plates m. p. 182.4–183.6° (corr.).

Two solutions containing 10 mg. of estrone and equilin per ml., respectively, were prepared by dissolving the calculated amount of the esters in carbon disulfide. Because of its more limited solubility, a solution containing 5 mg. of equilenin per ml. of carbon disulfide was prepared from the equilenin ester. Graphs of the absorption spectra of the three solutions were made using a 0.5-mm. cell for the estrone and equilin solutions and a 1.0-mm. cell for the equilenin solution. Significant differences

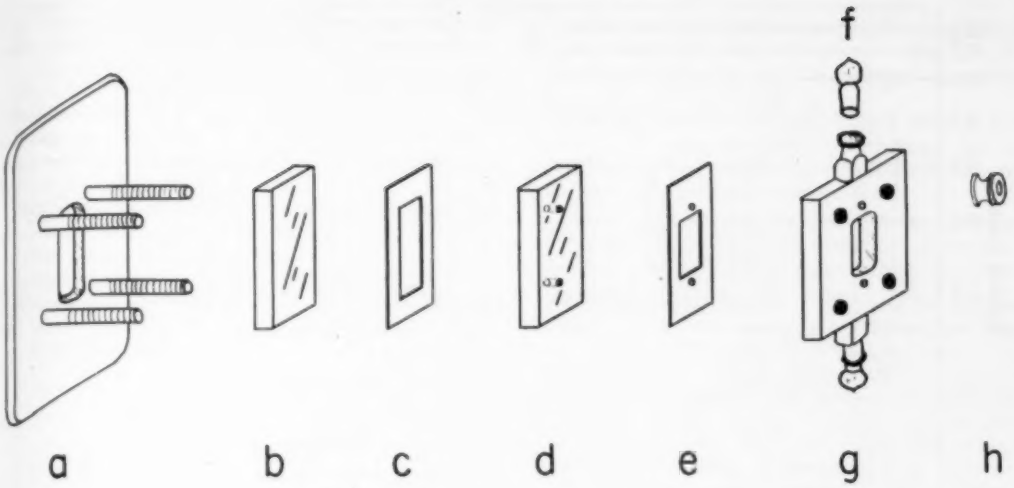

Fig. 1.—Details of liquid absorption cell.

a, back plate.
b, polished salt plate.
c, polyethylene spacer.
d, polished salt plate with 2 drilled holes.
e, polyethylene gasket.
f, glass stopper made from hypodermic syringe.
g, front plate (small holes in face connect with hypodermic needle adaptor); hypodermic syringe is used for filling cell.
h, nut

in these spectra occur between 8.5 and 14.0 μ as may be seen in Fig. 2. Examination of these curves showed that several combinations of wave lengths could be chosen for the analysis of the ternary mixture. Of these the following appeared to be most suitable: equilenin 10.45 μ, estrone 10.88 μ, and equilin 10.96 μ.

Three series of solutions of the ketosteroids in carbon disulfide were prepared from the calculated amounts of the esters so as to contain:

(*a*) 2 to 10 mg. estrone per ml.
(*b*) 2 to 10 mg. equilin per ml.
(*c*) 1 to 5 mg. equilenin per ml.

The optical density of each of these solutions, relative to a carbon disulfide blank, was measured at 10.45, 10.88, and 10.96 μ, using 0.5-mm. cells. These data are plotted in the three charts in Fig. 3. Adherence to Beer's law is shown by the straight line relationship between optical density and concentration that exists for each solution at each wave length. These graphs are used as a basis for the working curves for the determination of estrone, equilin, and equilenin.

each dissolved in exactly one ml. of carbon disulfide. The optical density of each solution, relative to a reagent blank, was determined using 0.5-mm. cells at 10.45, 10.88, and 10.96 μ. These data, along with the optical densities of solutions of the corresponding pure ester, are compared in Table I. These results show that the solutions prepared by the proposed method have absorptions practically identical with those of solutions of the pure crystalline esters.

The accuracy of the results obtained in the following method will depend, in part, on the care used in locating the absorption bands, and in the making of the optical density measurements. The position of the absorption bands reported herein should be re-determined with the instrument to be used for the analysis. These bands should be checked at frequent intervals as spectrophotometer calibration may change with temperature, vibration, etc. It must be emphasized that the same pair of cells should be used in making the working curves and for obtaining the analytical data. Optical density measurements made at the same wave length should be made using as near the same slit width as practical. All optical density measurements should be

TABLE I.—COMPARISON OF THE OPTICAL DENSITIES OF SOLUTIONS PREPARED ACCORDING TO THE METHOD AND FROM THE CRYSTALLINE ESTERS

	Estrone 10 Mg./Ml.		Equilin 10 Mg./Ml.		Equilenin 5 Mg./Ml.	
	By Proposed Method	Crystalline Ester	By Proposed Method	Crystalline Ester	By Proposed Method	Crystalline Ester
$E_{10.45}$	0.062	0.059	0.105	0.100	0.205	0.200
$E_{10.88}$	0.518	0.520	0.162	0.160	0.046	0.046
$E_{10.96}$	0.230	0.235	0.370	0.376	0.036	0.036

Ten milligrams each of estrone and equilin and 5 mg. of equilenin were esterified as directed under the method below and the resulting three esters were the average of at least three readings.

Apparatus.—A spectrophotometer suitable for measuring optical density in the infrared spectrum

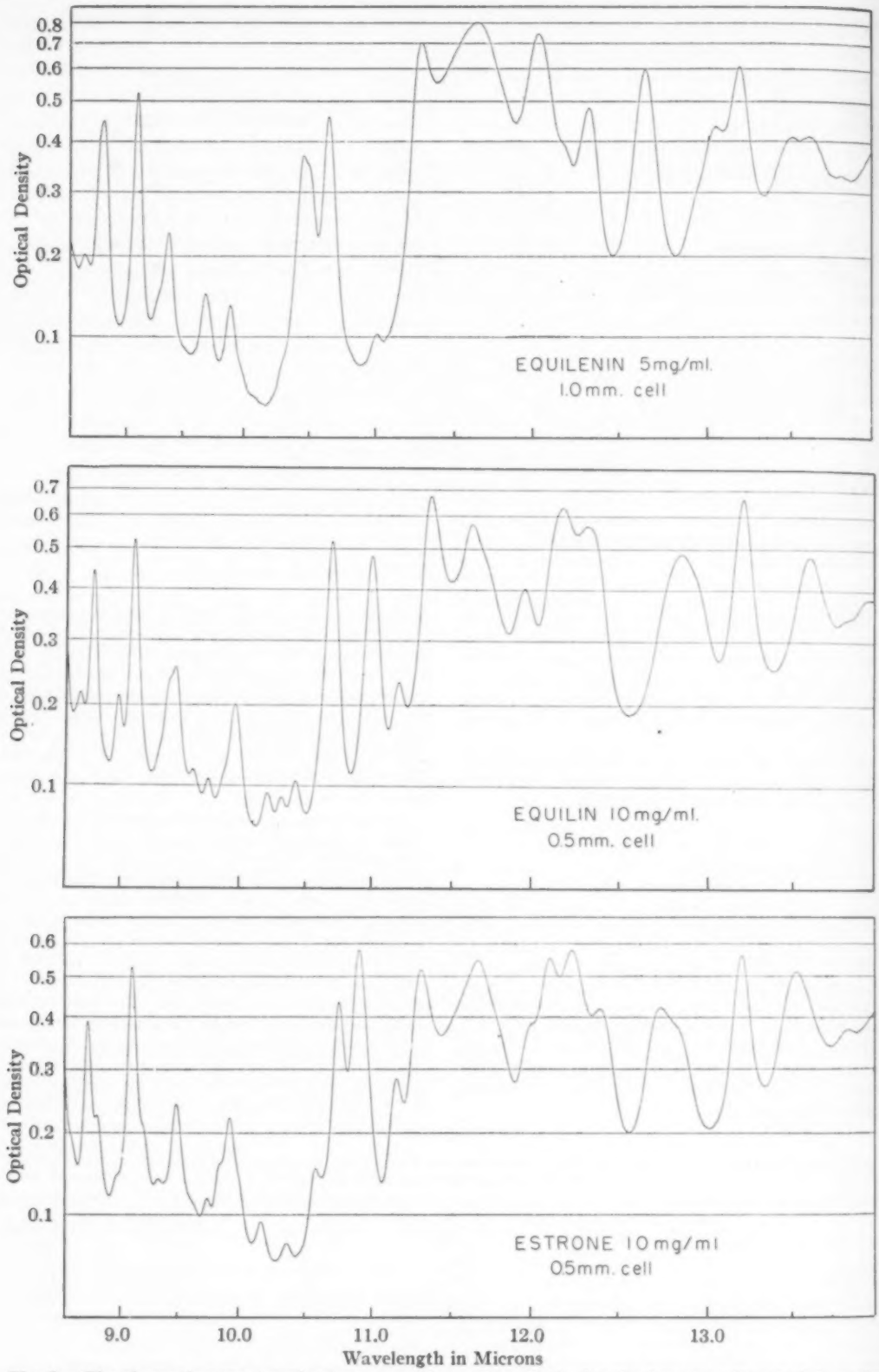

Fig. 2.—The absorption spectra of estrone, equilin, and equilenin (as the benzenesulfonates) in carbon disulfide solution. Concentrations are expressed in terms of the uncombined ketosteroid. (The strong absorption band at 11.7 μ is due to the carbon disulfide solvent.)

The instrument should be sufficiently free of stray light error to produce straight-line concentration curves under the conditions used in the method.

Two 0.5-mm. liquid absorption cells with sodium chloride windows.

Reagents—Pyridine A. R. grade distilled over solid KOH in an all-glass apparatus and stored in a glass-stoppered bottle.

Benzenesulfonyl chloride (redistilled in an all-glass vacuum distillation apparatus).

Chloroform—U. S. P. grade.

Carbon Disulfide—A. C. S. Reagent grade.

Standards.—Estrone U. S. P. Reference Standard, standard solution prepared to contain 0.5 mg./ml. in 95% alcohol.

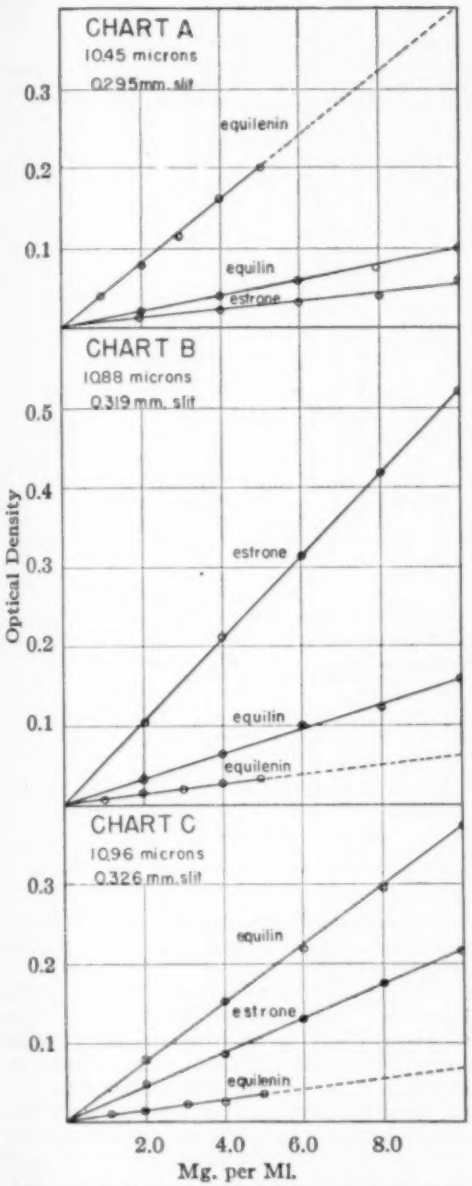

Fig. 3.—Plot of optical density against concentration for estrone, equilin, and equilenin (as the benzenesulfonates) in carbon disulfide solution at 10.45, 10.88, and 10.96 μ.

Equilin, standard solution prepared to contain 0.5 mg./ml. in 95% alcohol.

Equilenin, standard solution prepared to contain 0.5 mg./ml. in 95% alcohol.

Working Curves.—Measure from a burette into 25-ml. glass-stoppered Erlenmeyer flasks the required amounts of standard solution to prepare the three series of standards shown below:

Series 1—2.0, 4.0, 6.0, 8.0, and 10.0 mg. estrone.
Series 2—2.0, 4.0, 6.0, 8.0, and 10.0 mg. equilin.
Series 3—1.0, 2.0, 3.0, 4.0, and 5.0 mg. equilenin.

Evaporate each solution to dryness on a steam bath and dry the residue one hour in a vacuum desiccator. By the procedure described in the method, convert each standard to the benzenesulfonyl ester and dissolve in exactly 1 ml. carbon disulfide. Determine the optical density of each solution, relative to the reagent blank, at 10.45, 10.88, and 10.96 μ. From these data prepare three charts as shown in Fig. 3, plotting the optical densities of each series of standards against concentration.

Method.—Transfer up to 10 mg. (preferably 5–10 mg.) of the ketosteroids[1] to a 25-ml. glass-stoppered Erlenmeyer flask, dissolve in 2 ml. dry pyridine, add 0.25 ml. benzenesulfonyl chloride and stopper tightly. Mix gently and allow to stand overnight at room temperature. Dilute the contents of the flask with a few milliliters of water and transfer to a 125-ml. separatory funnel, restricting the total volume of water in the mixture to 25–30 ml. Rinse the flask with 15 ml. of chloroform and add to the funnel. Shake the funnel vigorously for one minute and allow the two layers to separate completely. Transfer the chloroform layer to a second 125-ml. separatory funnel. Repeat the extraction with two additional 15-ml. portions of chloroform adding each to the second funnel. Wash the combined chloroform extracts with 10 ml. water and filter through a pledget of cotton wet with chloroform into a small beaker. Extract the wash water with an additional 5 ml. chloroform, filter through the cotton, and add to the main chloroform extract. Evaporate the combined chloroform extract to dryness on a steam bath in a current of air. Dissolve the residue in 5 ml. chloroform and evaporate again to dryness. Dissolve the residue in about 2 ml. chloroform and transfer to a 15 x 50-mm. glass-stoppered weighing bottle with the aid of several small portions of chloroform. Carefully evaporate the solution to dryness on a steam bath using a current of air. Dry the residue in a vacuum for one hour to remove traces of pyridine. Using a 1-ml. graduated pipette, carefully add a measured quantity of carbon disulfide to produce a concentration of about 5–10 mg. of ketosteroids per ml. Stopper the bottle immediately and dissolve the residue by gentle mixing.[2] Prepare a reagent blank solution following the procedure directed above. Determine the optical density of the sample solution relative to the blank at 10.45, 10.88, and 10.96 μ.

[1] The ketosteroid residue obtained in the analysis of oil solutions of estrogens using Girard Reagent T (5) can be analyzed for estrone, equilin, and equilenin without further purification. Commercial samples of Girard Reagent T should be recrystallized from absolute alcohol and dried in a vacuum before being used in this procedure. It is also recommended that A.C.S. special grade 99.9% glacial acetic acid be used.

[2] Experience has shown that the occasional appearance of undissolved material is not due to incomplete solution of the ester. However, samples containing almost pure equilenin may dissolve with some difficulty.

Graphical Approximation Calculation

First Approximation.—From the optical density of the sample solution obtained at 10.45 μ, determine the concentration of equilenin from the equilenin working curve in Chart A. Using the same procedure, determine the approximate estrone concentration from the optical density of the sample solution obtained at 10.88 μ and the estrone working curve in Chart B, and determine the approximate concentration of equilin from the optical density of the sample solution obtained at 10.96 μ and the equilin working curve in Chart C. These approximate concentrations will all be too high because at each of these wave lengths the absorption obtained is due to all three components.

Second Approximation.—Using the concentration of estrone obtained in the first approximation determine the optical density due to estrone at 10.45 μ from the estrone working curve in Chart A, and using the concentration of equilin obtained in the first approximation determine the optical density due to equilin at 10.45 μ from the equilin working curve in Chart A. Subtract the sum of these optical densities from the optical density of the sample solution at 10.45 μ. Using this corrected optical density, determine the corrected concentration of equilenin from the equilenin working curve in Chart A. Using the corrected concentration of equilenin, determine the optical density due to equilenin at 10.88 μ from the equilenin working curve in Chart B, and using the concentration of equilin obtained in the first approximation, determine the optical density due to equilin at 10.88 μ from the equilin working curve in Chart B. Subtract the sum of these optical densities from the optical density of the sample solution at 10.88 μ. From this corrected optical density, determine the corrected concentration of estrone from the estrone working curve in Chart B. Using the corrected concentration of equilenin, determine the optical density due to equilenin at 10.96 μ from the equilenin working curve in Chart C, and using the corrected concentration of estrone, determine the optical density due to estrone at 10.96 μ from the estrone working curve in Chart C. Subtract the sum of these optical densities from the optical density of the sample solution at 10.96 μ. From this corrected optical density determine the corrected concentration of equilin from the equilin working curve in Chart C.

These corrected values of the estrone, equilin, and equilenin concentrations will usually be too low since the use of the approximate concentrations has led to overcorrection of the sample solution optical densities. Using the corrected concentrations, repeat the calculation as directed in the second approximation above. Repeat this process until subsequent approximations make little or no change in the concentration of each ketosteroid.

TABLE II.—RESULTS OF ANALYSES BY THE PROPOSED METHOD[a]

Sample	Mg. Estrone		Mg. Equilin		Mg. Equilenin	
	Added	Found	Added	Found	Added	Found
1	5.6	5.6	3.0	3.1	1.5	1.7
2	3.3	3.3	3.3	3.1	3.3	3.4
3	5.0	5.0	2.5	2.5	2.5	2.7
4	9.0	9.1	0.5	0.7	0.5	0.5
5	0.0	0.0	5.0	5.0	5.0	4.8
6	5.0	4.9	5.0	5.3	0.0	0.0
7	4.0	3.6	3.2	3.4	2.8	2.7
8	2.8	2.6	3.2	3.1	4.0	4.0
9	3.0	2.9	1.0	1.0	4.0	4.2
10	4.0	4.0	1.5	1.5	4.0	4.5
11	7.5	7.4	0.0	0.0	2.2	2.2
12	9.3	9.4	0.0	0.0	0.0	0.1
13	6.0	6.0	2.0	2.1	2.0	1.9
14	8.0	7.8	1.0	1.0	1.0	0.9
15	7.0	7.0	0.0	0.2	1.5	1.6
16	1.0	1.0	2.0	2.1	5.3	5.3
17	6.5	6.4	0.0	0.2	0.0	0.0
18	2.0	2.0	2.0	1.6	2.3	2.4
19	4.0	3.9	1.0	1.2	1.0	1.0
20	6.0	5.9	2.0	2.0	2.0	2.1

[a] Each result represents a single determination.

TABLE III.—RECOVERY OF KETOSTEROIDS ADDED TO COMMERCIAL SAMPLES OF ESTROGENS IN OIL

Sample		Mg. Estrone per 5 Ml.		Mg. Equilin per 5 Ml.		Mg. Equilenin per 5 Ml.	
		Added	Recovered	Added	Recovered	Added	Recovered
A	1	2.5	2.5	2.5	2.5	...	...
	2	2.5	2.5	...	...	2.5	2.5
	3	...	...	2.5	2.4	2.5	2.4
	4	1.6	1.5	1.6	1.5	1.6	1.5
B	1	2.5	2.4	2.5	2.5	...	...
	2	2.5	2.5	...	...	2.5	2.5
	3	...	...	2.5	2.4	2.5	2.4
	4	1.6	1.6	1.6	1.6	1.6	1.5

DISCUSSION

A series of mixtures of the three ketosteroids was

prepared and analyzed by the above method. These results are shown in Table II.

Five 5-ml. portions were taken from each of two commercial samples labeled, "A solution of estrogenic hormones derived from gravid mare's urine in sesame oil... 10,000 I. U. per cc." One portion of each sample was analyzed for total ketosteroid content by the method of Carol and Rotondaro (4). The ketosteroid residues obtained were then analyzed by the proposed method. Varying amounts of estrone, equilin, and equilenin, dissolved in alcohol, were then added to each of the other four portions. After removing the alcohol by evaporation on a steam bath, each portion was analyzed as above. These results are shown in Table III.

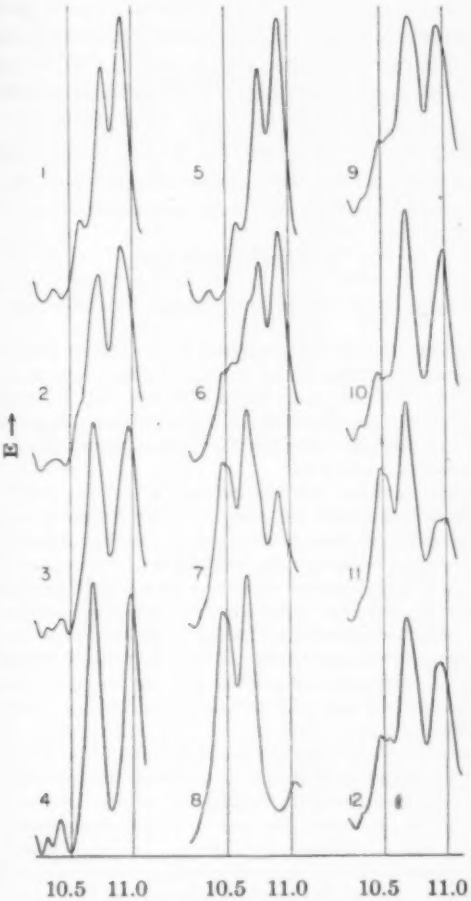

Fig. 4.—The effect of composition on the shape of the absorption spectra of ketosteroid mixtures in the 10.0–11.0 μ region.

1, 5, 100% estrone.
2, 66% estrone, 33% equilin.
3, 33% estrone, 66% equilin.
4, 100% equilin.
6, 66% estrone, 33% equilenin.
7, 33% estrone, 66% equilenin.
8, 100% equilenin.
9, 60% estrone, 20% equilin, 20% equilenin.
10, 20% estrone, 60% equilin, 20% equilenin.
11, 20% estrone, 20% equilin, 60% equilenin.
12, 33% estrone, 33% equilin, 33% equilenin.

In the routine analysis of mixtures of ketosteroids by the proposed method, it was found that a record of the 10.0–11.5 μ region of the absorption spectrum was very useful as a check on the results and as a means of detecting occasional contamination. These records were easily made at the time the optical densities were measured. With experience it was possible to predict fairly accurately the composition of the sample from the shape of its absorption curve. A series of these curves, made from samples of varying composition, is given in Fig. 4. These curves can be used for the rough estimation of the composition of a mixture of the ketosteroids when samples of pure estrone, equilin, and equilenin are unavailable.

SUMMARY AND CONCLUSIONS

1. The infrared absorption spectra of solutions of the benzenesulfonyl esters of estrone, equilin, and equilenin in carbon disulfide have been determined. These spectra show considerable differences in the 8.5–11.5 μ region.

2. A quantitative infrared spectrophotometric method of analysis, based on these differences, is given in detail. The method is relatively simple and should be entirely satisfactory for the routine analysis of commercial estrogenic hormone preparations.

3. Results of analysis of twenty control samples are given. Good results were obtained with mixtures containing as little as 5 per cent of one or more components.

4. Examples of the absorption curves of various mixtures of the three compounds are shown.

5. Varying amounts of ketosteroids were added to portions of two commercial samples of natural estrogens in oil. Analysis by the proposed method, after isolation of total ketosteroids with Girard Reagent T (4), showed satisfactory results.

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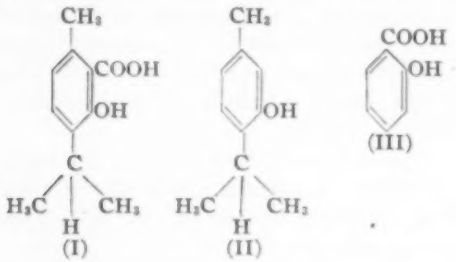
Preparation and Inhibitory Properties of Derivatives of *o*-Thymotinic Acid*

By JOHN P. STREET,† CARL E. GEORGI,‡ and PAUL J. JANNKE§

Directions for the preparation of derivatives of *o*-thymotinic acid are given along with the results of the analyses of the compounds. Phenol coefficients and fungicidal tests were run on the derivatives. Outstanding inhibitory properties are attributed to the bismuth, mercury, and silver salts and the methenamine complexes.

THE STRUCTURAL relationship of *o*-thymotinic acid (I) to thymol (II) and to salicylic acid (III) suggests that the acid should possess properties similar to those of both related compounds. Thymol has long been prominent as a bactericide



and vermicide, while salicylic acid and its derivatives are well known as analgesics, antipyretics, and antiseptics. It is logical, therefore, to anticipate considerable parallel activity from *o*-thymotinic acid. Although *o*-thymotinic acid has been known since 1860, the literature contains but a single reference to a potential application of it. Spallino and Provenzal (1) reported that aqueous solutions of both the free acid and its sodium salt possess marked bactericidal properties, but they offered no experimental evidence.

The preparation of *o*-thymotinic acid has been reported by several investigators (2–7) with yields ranging from 40 to 68 per cent. Recorded melting points vary between 120 and 127°. Rosati (8), who conducted crystallographic measurements on the compound, found that it belongs to the monoclinic system, with refractive indices corresponding to: $a:b:c = 1.0724:1:0.9039$.

The several derivatives which have been prepared and described have not been considered from the standpoint of their applications to pharmaceutical practice, although it would appear that among them are some which have definite potentialities. This report marks the beginning of a study of *o*-thymotinic acid derivatives in the hope of disclosing several which have therapeutic value. The derivatives mentioned are for the most part simple compounds which were prepared, analyzed, and subjected to certain microbiological tests *in vitro*. The phenol coefficient was used as a criterion of their bactericidal properties while their fungistatic activity was determined by the cup-plate technique.

EXPERIMENTAL

Chemistry of Thymotinic Acid and Its Salts

Preparation of *o*-Thymotinic Acid.—*o*-Thymotinic acid is best prepared according to Sondern's modification (9) of the Spallino-Provenzal method, wherein thymol dissolved in boiling xylene is treated with metallic sodium and dry carbon dioxide gas at atmospheric pressure.

The reaction was conducted in a 5-L., triple-necked flask, equipped with a stirrer, mercury seal, condenser, and an inlet tube for carbon dioxide. The gas train included a sulfuric acid drying bottle and a mercury relief valve. The reaction product has a tendency to accumulate in the inlet tube, so the relief valve was inserted to avoid excessive back pressure in the gas train. When the tube was clogged, as evidenced by bubbling in the valve, it was replaced by a new one without interrupting the reaction.

Two hundred fifty grams of thymol (1.66 moles) was dissolved in 2.5 L. of dry xylene, and the solution was heated to its boiling point in an oil bath. When this temperature was attained, stirring was begun and dry carbon dioxide was bubbled through the solution at a slow, uniform rate. Over a period of three hours, 75 Gm. (3.25 moles) of granulated sodium was introduced in teaspoonful (plastic) quantities. After the last portion of sodium had been added, the reaction mixture was stirred and heated for two hours longer. Then the mixture was allowed to cool and to stand overnight under an atmosphere of carbon dioxide. After decanting the supernatant xylene, the reaction mixture was transferred to a large beaker where the unreacted sodium was carefully decomposed with 75 cc. of isopropyl alcohol added in small quantities with a minimum of stirring. When no unreacted sodium remained, 1 L. of water was added and the mixture was stirred thoroughly.

Following the separation of the two immiscible liquids, the aqueous layer was filtered through Celite.

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Upon acidifying the filtrate with hydrochloric acid, the *o*-thymotinic acid separated in crystalline form. It was collected on a suction filter and washed with water until the washings were neutral to litmus. In this manner, *o*-thymotinic acid was produced in yields up to 71.1%.

In order to ascertain the ideal conditions for the optimum yield, the factors concerned were varied in accordance with the listings in Table I.

Disodium *o*-Thymotinate.—It is understood that replacement of the carboxylic hydrogen and the phenolic hydrogen by sodium tends to reduce the activity of salicylic acid (10). Nevertheless the disodium salt of *o*-thymotinic acid was prepared to compare the bacteriological action of the basic and the neutral salts. One-tenth mole (19.42 Gm.) of *o*-thymotinic acid was dissolved in the theoretical amount (100 cc.) of 2 *N* sodium hydroxide solution.

TABLE I.—FACTOR VARIANCE IN ACID PREPARATION

Lot	Time, Hr.	Sodium Gm.	Sodium Moles	Thymol Gm.	Thymol Moles	Xylene, Cc.	Thymotinic Acid, Gm.	Per Cent Yield
1	5	80	3.48	200	1.33	3000	115.5	44.6
2	7¾	80	3.48	200	1.33	3000	175.0	67.5
3	8½	80	3.48	200	1.33	2500	180.0	69.5
4	6	75	3.25	250	1.66	2500	230.0	71.1

The acid so prepared was light brown in color and it was purified by fractional precipitation from a solution of the ammonium salt. The lighter colored fractions, melting at 124°, were combined for recrystallization, and the dark colored fractions were redissolved in ammonium carbonate solution from which the free acid was reprecipitated fractionally. Since the acid still retained an odor of thymol, it was purified by recrystallization. The common organic solvents such as alcohol, chloroform, and benzene were found to be unsuitable for the recrystallization of *o*-thymotinic acid because of the higher order of solubility. Large, well-formed crystals could be obtained, but the recoveries were low and the acid contained traces of thymol. When benzene was used as a solvent, the crystals bore the odor of it for a long time.

By dissolving *o*-thymotinic acid in hot 50% acetic acid and filtering through Norite, fine needle-like crystals were obtained in excellent yields. The crystals were devoid of thymol, but even after thorough washing with water, they retained the odor of acetic acid. On occasion the colorless product melted as low as 121°, but after standing in the air for several days the acetic acid was dissipated and the melting point rose to 125°. The crystals can be freed from traces of acetic acid by heating for a few hours at 60°, raising the melting point to 126°.

Sodium (mono) *o*-Thymotinate.—The normal salt of thymotinic acid was prepared by titrating a sample of the acid weighing 19.42 Gm. with normal sodium hydroxide solution in the presence of phenolphthalein T. S. At the end point, the pink color of the indicator was discharged by adding a crystal of the acid. The solution was concentrated at 60° until crystallization began. Upon cooling, the sodium salt was obtained. It was redissolved in a minimum of hot water, treated with charcoal, and allowed to recrystallize. It melted with slight decomposition at 114°. The normal sodium salt of *o*-thymotinic acid is very soluble in water, alcohol, and ether, and slightly soluble in benzene.

The sodium content, determined according to the U. S. P. method for assaying sodium benzoate, was found to be 9.83%. Calculated for:

$C_{10}H_{12}(OH)COONa.$: 10.63%
$C_{10}H_{12}(OH)COONa.H_2O$: 10.20%
$C_{10}H_{12}(OH)COONa.1\frac{1}{2}H_2O$: 9.83%
$C_{10}H_{12}(OH)COONa.2H_2O$: 9.49%

The resulting solution was concentrated at 60° to the point where crystals separated. Upon cooling the solution, the crystalline mass (23.8 Gm. representing 100.0% yield) of disodium thymotinate was obtained. When recrystallized from 70% alcohol, following treatment with charcoal, it melted at 108°. It was very soluble in water and alcohol, but slightly soluble in ether and benzene.

The sodium content was found to be 17.9%, calculated for:

$C_{10}H_{12}(ONa)COONa$: 19.3%
$C_{10}H_{12}(ONa)COONa.H_2O$: 18.5%
$C_{10}H_{12}(ONa)COONa.1\frac{1}{2}H_2O$: 17.9%
$C_{10}H_{12}(ONa)COONa.2H_2O$: 17.3%

Silver *o*-Thymotinate.—The silver salt was precipitated in the dark by dissolving 5 Gm. of *o*-thymotinic acid in 15 cc. of a solution containing 1.3 Gm. of ammonium carbonate and adding to this 33 cc. of 17% silver nitrate solution. After the mixture stood in a cool dark place for 24 hours, the precipitate was filtered off and washed with water, alcohol, and ether. When dried, the white powder melted at 161°. The yield was 7.5 Gm., corresponding to 98.2%. The silver content, determined as the chloride, was found to be 35.73%; calculated for $C_{10}H_{12}(OH)COOAg$, 35.83%. Silver *o*-thymotinate is insoluble in water, very slightly soluble in ether and benzene, but dissolves in alcohol to the extent of 0.25 Gm. per 100 cc.

Magnesium *o*-Thymotinate.—The normal magnesium salt was prepared by adding an aqueous solution of 6.7 Gm. of sodium *o*-thymotinate in 45 cc. of water to 1.64 Gm. of magnesium chloride dissolved in 50 cc. of water. The salt began to separate immediately, with further precipitation occurring when the mixture was held at 10° for four hours. Recrystallization from hot water, following treatment with charcoal, yielded 6.8 Gm. of fine, white, flake-like crystals melting at 99°. The yield corresponded to 96.6%. The magnesium content corresponded to 6.09%; calculated for $(C_{10}H_{12}(OH)COO)_2Mg$, 5.92%.

Magnesium thymotinate is soluble to the extent of 0.76 Gm. per 100 cc. of water; 7.02 Gm. per 100 cc. of alcohol; soluble in ether, very slightly soluble in benzene.

Calcium *o*-Thymotinate.—Normal calcium *o*-thymotinate was prepared by adding 50 cc. of an

aqueous solution of sodium thymotinate (6.7 Gm.) to 3.4 Gm. of monohydrated calcium chloride in 50 cc. of water. After standing for 96 hours in the cold, the calcium salt was filtered off and recrystallized from hot, 50% aqueous-alcohol, after treatment with charcoal. The yield was 4.54 Gm., corresponding to 68.7%. The white, flaky crystals decomposed without melting between 235 and 240°.

The calcium content was found to be 9.37%; calculated for $(C_{10}H_{12}(OH)COO)_2Ca$, 9.39%. Calcium *o*-thymotinate shows the following solubilities: 2.66 Gm. per 100 cc. of water; 9.24 Gm. per 100 cc. of alcohol; extremely soluble in ether and benzene.

Barium *o*-Thymotinate.—The normal barium salt of *o*-thymotinic acid was prepared by pouring a solution of 14.2 Gm. of barium chloride dihydrate in 50 cc. of water into a solution of 25 Gm. of sodium *o*-thymotinate in 25 cc. of water. The solution was concentrated by heating for two hours at 60° and then refrigerated at 10° for 24 hours. The white, square plate-like crystals so obtained were recrystallized from 20% alcohol, following treatment with Norite. The yield was 26.5 Gm., corresponding to 87.4%. The melting point was 210° with slight decomposition.

Barium *o*-thymotinate dissolves in water to the extent of 1.72 Gm. per 100 cc.; in alcohol, 26.48 Gm. per 100 cc.; slightly soluble in ether and insoluble in benzene. The barium content corresponded to 26.19 per cent while the calculated value for $(C_{10}H_{13}(OH)COO)_2Ba$ is 26.22 per cent.

Mercuric *o*-Thymotinate.—Mercuric *o*-thymotinate was prepared by adding a solution of 8.2 Gm. of mercuric chloride in 50 cc. of water to a solution of sodium *o*-thymotinate (13 Gm. in 50 cc. water). The white precipitate which formed immediately was allowed to stand for 24 hours before it was collected on a filter, and washed with water and ether. Ten and one-tenth grams (57.9%) of minute white crystals were obtained. They melted at 152°.

The mercury content, determined as sulfide, was found to be 34.7%. The calculated mercury content for $(C_{10}H_{12}(OH)COO)_2Hg$ is 34.2%. The solubilities are: 0.1 Gm. per 100 cc. of water; 0.76 Gm. per 100 cc. of alcohol; insoluble in both ether and benzene.

It should be noted that there is no analogy between mercuric *o*-thymotinate and mercuric salicylate, N. F. VIII. The latter is the basic salt type.

Zinc *o*-Thymotinate.—The normal zinc salt of *o*-thymotinic acid was prepared by adding a solution of 6.6 Gm. of zinc acetate in 30 cc. of water to 13.0 Gm. of sodium *o*-thymotinate in 50 cc. of water. The resinous precipitate which formed immediately was washed with warm water, and the residue was dissolved in ether. Upon evaporation of the ether, zinc *o*-thymotinate was deposited in large sheet-like crystals melting at 105°. The yield was 12.35 Gm., corresponding to 90.8%.

The zinc content, determined as the oxide, was 14.57% as compared with the theoretical 14.47% for $(C_{10}H_{12}(OH)COO)_2Zn$. Solubility in water is of the order 0.26 Gm. per 100 cc.; 8.26 Gm. per 100 cc. of alcohol; very soluble in ether but insoluble in benzene.

Cupric *o*-Thymotinate.—The normal copper salt was prepared by adding a solution of 14.7 Gm. of copper sulfate in 50 cc. of water to 25.4 Gm. of sodium *o*-thymotinate in 75 cc. of water. A dark brown precipitate formed immediately, but after vigorous stirring for a short time the color changed to bright green. Attempts to isolate the precipitate in the brown (cuprous?) form were unsuccessful. The cupric salt was collected on a filter, washed thoroughly with cold water, and dried. The yield was 26.35 Gm., corresponding to 99.8% of the theoretical. The green crystalline powder melted at 171°.

The copper content corresponded to 13.64%; calculated for $(C_{10}H_{12}(OH)COO)_2Cu$, 14.13%. Cupric *o*-thymotinate is soluble to the extent of 0.1 Gm. in 100 cc. of water; 1.16 Gm. in 100 cc. of alcohol. It is extremely soluble in ether, and while it is soluble in benzene, it appears to decompose.

Lead *o*-Thymotinate.—The lead salt was prepared by adding a solution of 3.5 Gm. of basic lead acetate in 10 cc. of water to a solution of 2.5 Gm. of sodium *o*-thymotinate in 25 cc. of water. A voluminous white precipitate formed immediately. After standing for 48 hours in the cold, it was collected on a filter, washed thoroughly with cold water and dried at 60°. The resulting white powder decomposed without melting at 210°. The yield was 4.61 Gm. (99.9%).

The lead content of lead *o*-thymotinate, determined as the sulfide, was 52.11%, as compared with the calculated percentage, 51.9, for $C_{10}H_{12}(COOPbO)$. The solubility of the lead salt in water was found to be 0.14 Gm. in 100 cc., and in alcohol 4.92 Gm. in 100 cc. The salt is soluble in ether, but insoluble in benzene. Apparently it is the basic salt.

Ferrous *o*-Thymotinate.—This salt was prepared by adding a solution of 2.35 Gm. of ferrous sulfate (dried) in 50 cc. of water to a solution of 6.9 Gm. of sodium *o*-thymotinate in 45 cc. of water, with vigorous stirring. A magenta-colored precipitate formed immediately. After standing in the cold for 48 hours the precipitate, now grayish black, was collected, washed with cold water, and dried. The crystals (3.42 Gm. corresponding to 50%) decomposed without melting at 103°.

The ferrous iron content, determined by the permanganate method, was 12.40%. Calculated iron content for $(C_{10}H_{12}(OH)COO)_2Fe$, 12.63%. Solubility of the ferrous salt in water is low, with 0.04 Gm. dissolving in 100 cc.; in alcohol, 4.96 Gm. dissolve in 100 cc. Extreme solubilities in both ether and benzene were noted.

Ferric *o*-Thymotinate.—The ferric salt of *o*-thymotinic acid was prepared by adding a solution of 2.8 Gm. of ferric chloride hexahydrate in 45 cc. of water to a solution of 6.7 Gm. of sodium *o*-thymotinate in 45 cc. of water. A black precipitate formed immediately. After standing in the cold for 48 hours it was collected on a filter, washed with water, and dried. The fine crystals, 6.5 Gm. or 99.8%, melted at 97° when tested in a bath preheated to 75°.

The iron content, determined by titration with standard sodium thiosulfate solution in the presence of an excess of potassium iodide (see under Ferric Ammonium Citrate, U. S. P.) (11), corresponded to 12.19%. The iron content calculated for $(C_{10}H_{11}(OH)COO)_3Fe$ is 8.79%. Several theories have been offered (12) for the possible structure of reaction products of ferric chloride and phenols, and in this instance the matter is further complicated by

the fact that a sodium salt was treated with the strongly acidic ferric chloride.

The compound designated ferric *o*-thymotinate is a complex mixture. Moreover, since it was shown in the preparation of the copper salt that thymotinic acid can function as a reducing agent, it is possible that the ferric salt contains some ferrous salt. Such a mixture is not detectable by the assay method employed which is a measure of ferric iron only.

The compound dissolved in water to the extent of 0.06 Gm. per 100 cc.; in alcohol, 3.16 Gm. per 100 cc.; extremely soluble in ether and benzene.

Aluminum *o*-Thymotinate.—The normal aluminum salt of *o*-thymotinic acid was prepared by slowly adding a solution of 6.0 Gm. of anhydrous aluminum chloride in 25 cc. of water to 25.0 Gm. of sodium *o*-thymotinate in 50 cc. of water. The voluminous white precipitate was separated, washed with water, and dried. The yield was 23.5 Gm. (99.5%). It was recrystallized from ether, following treatment with charcoal. Its melting point was not determined, but it remains unchanged up to 300°.

Aluminum *o*-thymotinate is soluble in ether and in benzene. In water, 0.08 Gm. dissolves in 100 cc., and in alcohol, 1.06 Gm. in 100 cc. The aluminum content was found to be 4.0%, while the calculated content for $(C_{10}H_{12}(OH)COO)_3Al$ is 4.04%.

Bismuth *o*-Thymotinate.—A bismuth salt was prepared by heating 2.0 Gm. of thymotinic acid with 0.9 Gm. of bismuth subcarbonate in an evaporating dish, with 10 cc. of water. Heating on a steam bath was continued until effervescence ceased. The reaction product was extracted with 25 cc. of alcohol and the alcoholic solution was filtered and set aside for crystallization to occur; 2.65 Gm. of light yellow crystals (98.1%) were obtained. They melted at 116°. While insoluble in water, the bismuth salt was shown to be extremely soluble in ether, alcohol, and benzene. The bismuth content, determined by ignition of the sample to bismuth trioxide, was found to be 4.0%; calculated for the normal salt $(C_{10}H_{12}(OH)COO)_3Bi$, 26.5%. On two different occasions, additional quantities of the bismuth compound were prepared, and upon analysis the bismuth content was found to be the same. Obviously the compound is not the normal bismuth salt. This is not unusual because bismuth is known to form numerous complexes of indefinite composition, for example bismuth subcarbonate, bismuth subsalicylate, and bismuth subgallate.

Mono-thymotinate-methenamine Sodium.—Twenty grams of sodium *o*-thymotinate and 13.0 Gm. of methenamine were dissolved in 56 cc. of water. Slow evaporation at 60° yielded an abundance of well-formed crystals melting at 140°. The compound salt had a pronounced affinity for water. The solubility in water was 54.1 Gm. per 100 cc.; in alcohol, 25.78 Gm. per 100 cc.; it was very soluble in ether and benzene.

Di-thymotinate-methenamine Sodium.—This compound salt was formed by combining 2 moles of mono-sodium thymotinate with 1 mole of methenamine. Six and nine-tenths grams of sodium thymotinate dissolved in 45 cc. of water were combined with 2.2 Gm. of methenamine in 15 cc. of water. The solution was concentrated at 60°, yielding minute crystals melting at 99.9°. It was extremely soluble in alcohol, soluble in ether, and insoluble in benzene. In water, its solubility was recorded as 41.7 Gm. per 100 cc.

Bactericidal and Fungistatic Properties of Thymotinic Acid and Its Salts[1]

Phenol Coefficients.—The method employed in determining the phenol coefficient was that of the Food and Drug Administration (13) using the F. D. A. strain of *Staphylococcus aureus* at a test temperature of 37.5°. The solvent used for making the salt dilutions for these tests was 20% ethanol. Control phenol coefficients on the solvent bases indicated no inhibition of *S. aureus*.

The experimental data are summarized in Table II.

TABLE II.—PHENOL COEFFICIENTS OF THYMOTINIC ACID AND SIXTEEN OF ITS SALTS

Salts	Coefficients
Thymotinic acid	1.67
Sodium (mono) thymotinate	1.30
Sodium (di) thymotinate	0.94
Magnesium thymotinate	4.72
Silver thymotinate	38.81
Calcium thymotinate	2.25
Barium thymotinate	1.08
Mercuric thymotinate	12.76
Zinc thymotinate	1.66
Aluminum thymotinate	6.94
Ferrous thymotinate	1.03
Ferric thymotinate	1.05
Lead thymotinate	1.05
Mono-thymotinate-methenamine sodium	0.72
Di-thymotinate-methenamine sodium	1.24
Bismuth thymotinate	6.49

Fungistatic Tests.—Investigations were conducted to determine the fungistatic properties of the salts of thymotinic acid by comparing the zones of inhibition against five different test organisms. They were *Candida albicans* (University of Nebraska strain), *Microsporum canis* (Duke University strain), *Epidermophyton floccosum* (Duke University strain), *Trichophyton purpureum* (T_8 Benham), and *Trichophyton mentagrophytes* (640), all fungi of significance in dermatophytoses in man.

The stock cultures were grown on Sabouraud agar slants at 28° for five to seven days. These cultures served as inocula for the plate inhibition tests. Twenty-five cubic centimeters of Sabouraud agar were introduced into a sterile clay-top Petri dish, allowed to solidify and then "seeded" with a water suspension of an organism spread uniformly over the agar surface by means of a sterile cotton swab.

The fungistatic activity of the various salts of thymotinic acid was determined with the compounds incorporated in both liquid and ointment preparations. When the base was a liquid, the "penicylinder" (or cup-plate) method was used, whereas with an ointment base, the agar depression-cup method (13) was employed to best advantage.

[1] The authors wish to acknowledge the technical assistance of Raymond F. Koubek on a portion of the microbiological investigations.

Solutions of the more soluble salts were tested by the first method while those with low degrees of solubility were employed in ointment form, using the latter technique. Liquid bases included 20% and 50% ethyl alcohol. The ointment base was a white ointment prepared according to the U. S. P. XIII (14).

Plates were incubated at 28° for seven days at which time the diameters of the zones of inhibition were measured and the areas of the zones calculated. The complete data are reported in Table III and the results with the most effective compounds graphically represented in Figs. 1, 2, 3, and 4. The zones of inhibition reported are the average of the inhibition zone minus the area occupied by the cylinder or agar depression cup. For control purposes, the plain ointment base and the alcoholic solutions were used. None of these exhibited any fungistatic activity when the salts of thymotinic acid were not incorporated in them.

TABLE III.—FUNGISTATIC ACTIVITY OF SALTS OF THYMOTINIC ACID

Salt	Dilution	Base[a]	Microorganism[b] 1	2	3	4	5
			Area of zone of inhibition in cm.[2]				
Mono-sodium	1–100	A	0.00	0.00	1.25	4.26	0.00
Di-sodium	1–100	A	8.25	0.00	0.00	1.36	0.00
Magnesium	1–200	B	10.91	4.00	1.50	7.62	1.73
Silver	1–10	C	0.00	0.00	0.00	0.00	0.00
Calcium	1–200	A	1.15	0.00	0.08	0.59	0.00
Barium	1–100	A	0.00	0.00	0.50	4.10	0.00
Mercuric	1–10	C	9.94	3.73	6.26	14.78	0.00
Zinc	1–100	C	0.00	0.00	0.00	0.00	0.00
Aluminum	1–200	B	0.42	1.79	1.46	5.52	0.00
Cupric	1–10	C	1.73	0.00	1.59	0.00	0.00
Ferrous	1–100	C	0.00	0.00	0.00	0.00	0.00
Ferric	1–100	C	0.00	0.00	0.00	0.00	0.00
Lead	1–20	C	0.00	0.00	0.00	0.00	0.00
Di-thymotinate methenamine	1–100	A	25.86	8.53	..	16.13	..
Mono-thymotinate methenamine	1–100	A	16.30	19.13	2.60	17.60	0.00
Bismuth	1–10	C	13.30	6.06	3.61	10.80	3.61

[a] Bases: A, 20% alcohol; B, 50% alcohol; C, plain ointment.
[b] Organisms: 1, *Epidermophyton floccosum;* 2, *Microsporum canis;* 3, *Trichophyton mentagrophytes;* 4, *Trichophyton purpureum;* 5, *Candida albicans.*

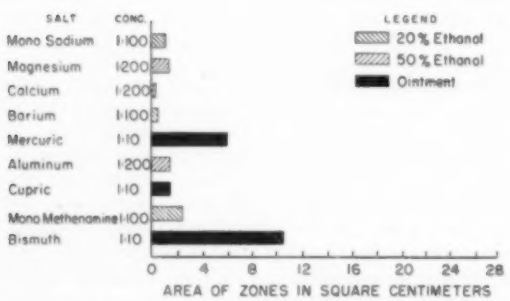

Fig. 1.—Zones of inhibition produced by salts of *o*-thymotinic acid against *T. mentagrophytes* (640).

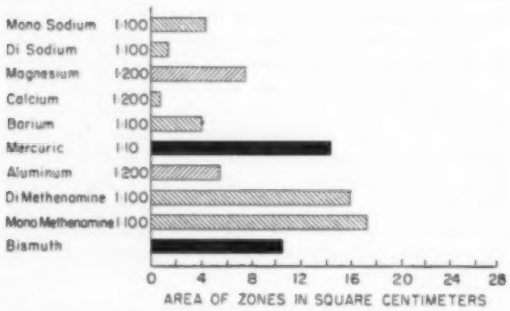

Fig. 2.—Zones of inhibition produced by salts of *o*-thymotinic acid against *T. purpureum* (T_8).

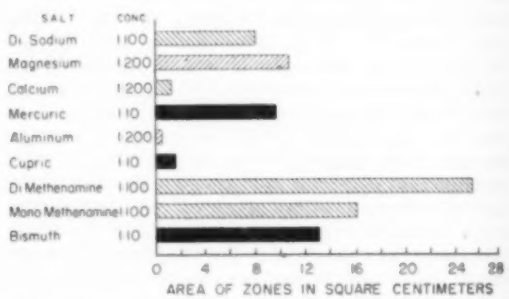

Fig. 3.—Zones of inhibition produced by salts of *o*-thymotinic acid against *E. floccosum* (Duke).

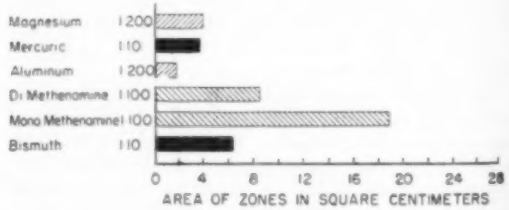

Fig. 4.—Zones of inhibition produced by salts of *o*-thymotinic acid against *M. canis* (Duke).

DISCUSSION

The results of the phenol coefficient tests indicate considerable variation in the effectiveness with which the different salts of thymotinic acid arrest the growth of *S. aureus* at 37.5°. The range is from 0.72 to 38.81. Derivatives which exhibit a coefficient in a range indicative of their potential value as topical disinfectants are the silver (38.8) and mercury (12.8) salts. The bismuth (6.5) and aluminum (6.9) salts are next in order with coefficients of about equal value but about one-half that of mercury. The other derivatives seemed to be of little or no value when compared to phenol as a standard of 1.0.

The fungistatic activity of thymotinic acid and its derivatives revealed their use as potential fungistatic agents. Of the microorganisms studied, *Candida albicans* proved to be the most resistant to the action of these chemotherapeutic agents, followed by *M. canis* and *T. mentagrophytes* in that order. *E. floccosum* and *T. purpureum* were the most susceptible to the compounds investigated.

Mono- and di-thymotinate methenamine appear to be the most active salts except for *T. mentagrophytes*. The greatest inhibitory effect of any compound on any of the five fungi studied was that exhibited by di-thymotinate methenamine on *E. floccosum*. On *M. canis*, the di compound was about 50% as effective as the mono derivative. On *T. purpureum*, the two salts were about equal in effectiveness and toward *T. mentagrophytes* the mono salt was disappointingly inactive.

The mercuric salt was especially effective against *T. mentagrophytes* where all other compounds failed but bismuth. The mercuric derivative was very active against *E. floccosum and T. purpureum*.

The bismuth salt was particularly inhibitory to *T. mentagrophytes* which organism proved resistant to all compounds save mercury. Bismuth gave a good account of itself in connection with the other organisms also, equaling the di- and mono-thymotinate methenamine salts in antagonizing *E. floccosum*..

The general inhibitory action of metals like bismuth and mercury is well known but the activity of magnesium is enlightening. That salt was fairly active in antagonizing both *E. floccosum* and *T. purpureum*.

Of little inhibitory value were the barium, aluminum, calcium, and sodium salts and of no fungistatic activity whatsoever were the zinc, silver, cupric iron, and lead derivatives.

SUMMARY

The chemistry of thymotinic acid and of sixteen salts is described.

The bactericidal activity and fungistatic value *in vitro* of these sixteen derivatives was studied.

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WHO MAKES IT?

The National Registry of Rare Chemicals, Armour Research Foundation, 33rd, Federal and Dearborn Streets, Chicago, Ill., seeks information on sources of supply for the following chemicals:

Fluoromalonic acid
Laurylphenoxyacetic acids
Polyvinylpyrrolidone
Dibenzofurane-3-sulfonic acid
β-(*m*-Hydroxyphenyl) ethylamine
Methionic acid and diphenyl ester
Divinyldichlorosilane
Phenyl isoxazoles
l-Galactose
l-Glucose
l-Mannose
Idose
Gulose
Talose
Allose
Altrose
Erythrose
Threose
Triglycollamic acid
2-Mercaptobenzoselenazole
Juniperic acid
N-Methyldiiodochelidamic acid

Colorimetric Assay of Digitoxin*

By ALEXANDER T. WARREN, FRANK O. HOWLAND, and LOUIS W. GREEN

A colorimetric method in which sodium beta-naphthoquinone-4-sulfonate (purified) is reacted with digitoxin in alkaline solution to form a purple color is proposed for the determination of digitoxin. The purple color quickly reaches a maximum and fades, but the addition of acetic acid at the time of maximum development changes the color to yellow and stabilizes it. The procedure involves no special equipment and may be adapted to any colorimeter or spectrophotometer by appropriate changes in the digitoxin concentration to suit the particular instrument. Data and tables are given to show results obtained with various samples of digitoxin and with several lots of tablets.

DIGITOXIN is becoming increasingly important in the treatment of certain heart conditions. Due to the expense and difficulties of the biological assay on this material, a rapid and simple chemical assay is desired for routine analysis. Bell and Krantz (1, 2) have reported a method based on the Baljet (3) reaction and using picric acid as the colorimetric reagent. Their procedure has been made the basis for Method I, Digitoxin Colorimetric Controls, in the U. S. P. XIII. James, *et al.* (4), have reported recently a method based on the Keller-Kiliani reaction, using ferric chloride in glacial acetic acid and concentrated sulfuric acid to develop a color. This procedure has been adopted as Method II, Digitoxin Colorimetric Controls in the U. S. P. XIII. Also, Anderson and Chen (5) have proposed a method in which digitoxin is reacted with *m*-dinitrobenzene to produce a blue color suitable for measurement in a photoelectric colorimeter.

Ehrlich and Herter (6) and Feigl (7, 8) have described the use of sodium β-naphthoquinone-4-sulfonate for the detection of reactive methylene groups. As digitoxin contains this grouping in the lactone ring of the aglycone digitoxigenin, its reaction with the reagent was tried and was found to produce a purple color in an alkaline solution. This is probably due to the formation of a paraquinoid condensation product. On acidification, the color is changed to a stable yellow which can be measured photometrically. We have used this reagent in the detection of digitoxin and to develop a method for the assay of digitoxin powders and tablets.

EXPERIMENTAL

Detection.—The procedure used is a modification of that outlined by Feigl (8) for the detection of reactive methylene groups. Dilute an alcoholic solution of the sample (0.1 to 1.0 cc.) to 2.5 cc. with water. Add 1 drop of 1% sodium β-naphthoquinone-4-sulfonate in 50% ethyl alcohol and 1 drop of 0.5 *N* NaOH. Observe after ten minutes. If a purple color is produced, add 1 drop of 2 *N* acetic acid. The yellow color developed on the addition of acetic acid is stable for several hours and may be extracted with butyl phthalate. This test will detect somewhat less than 50 micrograms of digitoxin.

Assay Procedure.—The following reagents are required:

1. A reagent solution containing 0.024% of sodium β-naphthoquinone-4-sulfonate and 0.024% of sodium sulfite in distilled water. This reagent solution is stable for at least one week.
2. An acetic acid solution containing 13% of acetic acid in aldehyde-free ethyl alcohol.
3. Aldehyde-free ethyl alcohol, prepared according to the U. S. P. XIII.
4. Chloroform, U. S. P. XIII.
5. Sodium hydroxide, 0.05 *N*.

A bath at 100° is required. A beaker containing mineral oil and placed on an adjustable hot-plate is sufficient for this purpose.

Transfer 10 cc. of a chloroform solution containing approximately 20 micrograms of digitoxin per cc. into a 25-cc. Erlenmeyer flask and evaporate the solution on a steam bath under a gentle stream of air. Add 0.1 cc. of chloroform and swirl the flask to wet all of the residue. Add 4 cc. of alcohol and 0.5 cc. of sodium hydroxide.

Place the flask to a depth of 1 cm. in the 100° bath. Exactly one minute later add as rapidly as possible (3 to 5 seconds) 1 cc. of the reagent solution and agitate slightly to produce a uniform solution. Exactly one and one-half minutes after the addition of the reagent solution, add 0.5 cc. of acetic acid solution with agitation. Immediately remove the flask from the bath, and cool under the tap. Transfer the contents of the flask into a 25-cc. volumetric flask and make up to the mark with alcohol.

Measure the per cent transmission of the solution within two hours with a photoelectric colorimeter such as the Pfaltz and Bauer Fluorophotometer. Adjust the instrument at 100% transmission with alcohol and use filters transmitting between 4200–4600 Å.

A blank is prepared in the same manner, omitting the digitoxin solution and starting with 0.1 cc. chloroform and 4 cc. of alcohol in a 25-cc. Erlenmeyer flask.

Standardization.—Dry about 50 mg. of the U. S. P. Digitoxin Reference Standard at 100° to constant weight. Dissolve in 250 cc. of chloroform. Transfer 5-, 7-, 10-, and 15-cc. portions each into

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100-cc. volumetric flasks and dilute each to the mark with chloroform. Transfer 10 cc. of the various solutions into 25-cc. Erlenmeyer flasks, and proceed as given under Assay Procedure. Prepare one blank with the four reactions and measure the per cent transmission of each.

Assay of Tablets.—Determine the total and average weight of 20 tablets, grind into a fine powder, and weigh out a sample approximately equal to the weight of 10 tablets. Add 20 cc. of chloroform to the sample, heat the mixture to boiling on a hot-plate, and filter the mixture through a fritted-glass filter, collecting the filtrate in a 50-cc. volumetric flask. Wash the residue several times with warm chloroform, collecting all filtrates in the volumetric flask. Adjust the combined filtrates to room temperature and make up to the mark with chloroform. Transfer 10 cc. of the solution into a 25-cc. Erlenmeyer flask and proceed as given under Assay Procedure. Perform in duplicate.

The above dilution applies to 0.1-mg. tablets; dilute the filtrates to 100 cc. in the assay of 0.2-mg. tablets.

Calculations.—Determine the $L_{1\text{ cm.}}^{1\%}$ value of the sample:

$$L_{1\text{ cm.}}^{1\%} = (2 - \log R)/CD$$

where R = per cent transmission of the sample,
C = concentration of the chloroform assay solution in per cent,
D = length of the light path through the cell.

Average the four $L_{1\text{ cm.}}^{1\%}$ values obtained from the standardization. If one of the values differs by more than 2% from the average, disregard that value and average the remaining three.

In the assay of a digitoxin powder, $L_{1\text{ cm.}}^{1\%}$ (sample)/$L_{1\text{ cm.}}^{1\%}$ (standard) × 100 = per cent digitoxin in the powder.

In the assay of tablets, $L_{1\text{ cm.}}^{1\%}$ (sample)/$L_{1\text{ cm.}}^{1\%}$ (standard) × average tablet weight = weight of digitoxin per tablet.

DISCUSSION

The Beer-Lambert law is followed over a concentration range of 10 to 40 micrograms per cc. of the chloroform assay solution. None of the excipients in the tablets tested gave a positive test for digitoxin. Lactose partially inhibited the formation of the purple color. Aldehydes were found to produce reagent blanks of increased color intensity. For this reason aldehyde-free alcohol was used. Other materials related to digitoxin which gave strong positive tests were Lanatoside A and Lanatoside C; weakly positive tests were given by Gitoxin and Lanatoside B; a negative test was given by digitonin.

Chloroform is used in preparing solutions for assay and as the extracting medium for tablets because of the high solubility of digitoxin in this solvent and because chloroform does not extract lactose from the powdered tablets, thereby preventing the interference of that material in the assay. Our experimental work has shown that the rapid extraction of tablets with boiling chloroform completely removes the digitoxin and makes unnecessary the lengthy overnight extractions previously reported.

The dilutions given in the various assay procedures were convenient for our purposes and produced colors of the required intensity for measurement on our instrument, a Pfaltz and Bauer Fluorophotometer. They may be changed to meet the needs of instruments of different sensitivity.

The 25-cc. Erlenmeyer flasks used in the assays should be of closely similar weights (within ±0.1 Gm.), the assumption being that flasks of equal weight and dimensions will have equal wall thickness and thereby allow equal rates of heating when the flasks are placed in the 100° bath. This precaution will allow close agreement on separate reactions run on aliquots of the same digitoxin solution.

The yellow color formed on the addition of the acetic acid solution is stable, reaches maximum intensity immediately, and fades only about 1% after two hours. This lack of a critical absorption measurement time is an advantage which this method has over other proposed methods. If a series of reactions are run, the absorptions can be measured at one time and do not have to be measured separately as each reaction is finished. The readings do not have to be timed; neither do several readings have to be made at intervals to determine the maximum intensity of a given reaction.

There are only two critical timing intervals during the reaction; the one wherein the reaction flask is heated for exactly one minute before the addition of the reagent solution, and the one and one-half minute interval after the addition of the reagent solution. Under the conditions of the reaction, the purple color reaches a maximum in one and one-half minutes, and soon begins to fade. The addition of the acetic acid at the end of that time arrests the reaction at the point of maximum development. At lower temperatures the color forms slowly, reaching a much lower maximum intensity before beginning to fade. Reproducible results could not be obtained at room temperature or at slightly higher than room temperatures. If the reaction was heated higher than 100°, the color development was too rapid for accurate timing.

The quantities of reagents used have been found by experiment to give the maximum color development under the conditions stated. The concentrations of reagents were selected for convenient addition from wide-orifice 1-cc. Mohr pipettes. The reaction proceeds better the higher the concentration of alcohol in the reaction mixture. Consistent with this fact, the volume of alcohol selected was the maximum permitted without precipitation of the alcohol-insoluble ingredients of the reagents. The addition of sodium sulfite to the reagent was found to yield lighter-colored blanks.

Two lots of sodium β-naphthoquinone-4-sulfonate were used in the experimental work. The first lot, which had been in stock for some time, gave satisfactory results. The second lot, recently purchased, produced erratic results. When purified according to the method given by Folin (9), the reagent was suitable for use.

Tables I and II show the results of assays on various tablets and powders, and indicate the reproducibility of the method.

TABLE I.—ASSAY OF TABLETS

Manufacturer	Tablet Lot No.	Mg. of Digitoxin/Tablet Label	Found	No. of Assays	Av. Deviation, ±%
A	1	0.2	0.197	4	1.4
A	2	0.2	0.189	3	1.8
A	3	0.1	0.095	3	3.1
A	4	0.2	0.183	3	0.4
B	5	0.1	0.092	3	1.4
C	6	0.2	0.197	3	3.0
D	7	0.2	0.196	3	0.6
E	8	0.1	0.092	3	0.5

TABLE II.—ASSAY OF DIGITOXIN POWDERS

Digitoxin Lot No.	Digitoxin Found, %	No. of Assays	Av. Deviation, ±%
1	92.9	3	1.9
2	92.0	3	0.3
3	97.9	3	1.3
4	108.9	10	2.3
5	96.9	12	1.4

All of the assay values in Tables I and II are with reference to the U. S. P. Digitoxin Reference Standard, which was assayed seventeen times with an average deviation of ±1.7%, and to which is assigned the value "100% digitoxin." Digitoxin Lot No. 4, Table II, is a specially purified sample of digitoxin prepared in this laboratory.

Tablet Lot No. 5, Table I, consisting of pink-colored tablets, appeared to extract colorless with chloroform. However, a small amount of the red dye was found to be present and produced a final reaction solution that was orange instead of bright yellow. Qualitative tests indicated that the dye was not changed in color by acids or alkalies Therefore, correction for the dye was made by evaporating to dryness an aliquot of the chloroform assay solution and dissolving the residue in a previously prepared reagent blank. Since this lot of tablets was the product of another manufacturer, the name of the dye was not known, and further study of its effect on the reaction could not be made.

SUMMARY

1. A color reaction for digitoxin, using sodium β-naphthoquinone-4-sulfonate, has been made the basis for its detection and determination.

2. The qualitative test is simple and will detect somewhat less than 50 micrograms.

3. The quantitative method is rapid and accurate, and requires no specialized equipment.

4. Assays of digitoxin powders and tablets have been given to indicate the reproducibility of the method.

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The Stability and Activity of Salivary Amylase*

By CHARLES W. BAUER† and WILLIAM F. MARTIN

Several factors influencing salivary amylase activity have been investigated and the results of the study are reported.

THE FUNCTION of salivary amylase as a digestive enzyme is well known. It is not so well known that if carbohydrates are not properly masticated, so that the salivary amylase is brought into intimate contact with them, the subject frequently suffers from severe dyspepsia. There is ample evidence, however, that subjects with low stomach acidity frequently suffer gastric distress from carbohydrate fermentation if the carbohydrates that are hydrolyzed by salivary amylase are not digested in the mouth or if, because of improper mastication, an insufficient amount of salivary amylase is carried to the stomach with the food to complete this reaction.

During an investigation of the salivary amylase activity of students over a period of many years,[1] it was observed that a number of the subjects afflicted with skin eruptions (and in one case definitely diagnosed as acne) had either less

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[1] Record of Observations of Experiments by C. W. Bauer, and classes in Biochemistry, 1940–1947 (unpublished).

salivary amylase than the normal subjects, or a very sluggish salivary amylase that was resistant to the activating effect of sodium chloride. It was thought that the cause of these skin eruptions was a lack of digestive enzymes, a deficiency that probably caused the formation of abnormal digestive products that, in some way, were responsible for these skin eruptions. Since the digestion of food in the human subject starts in the mouth, and since the activity of salivary amylase can be tested *in vitro*, an investigation was made in which the activity of this enzyme in normal subjects was compared with its activity in subjects with skin eruptions.

The salivary amylase acting on starch and glycogen converts these carbohydrates into soluble hydrolytic products, which include soluble starch, erythrodextrin, achrodextrin, and maltose. In this experimental work the progress of digestion was followed by testing with a dilute solution of iodine.

The activity of salivary amylase is noticeably influenced by temperature changes, hydrogen-ion concentration, and electrolytes. In order to insure reproducible results, the digestion mixtures were kept at 40° and were buffered to a *p*H of 6.8. Sodium chloride was the activator used in these experiments.

EXPERIMENTAL

Variations in the Saccharogenic Power of Human Saliva.—Walker and Shepherd (1) say: "There is a marked variation in the saccharogenic power of the saliva of different individuals on mixed diets, when the samples are collected at the same time of day. There is an appreciable variation in the activity of samples collected from the same individual on successive days. The ingestion of food at mealtime is followed by increased activity of the salivary amylase. This increase is greatest with breakfast, least with the evening meal. There is usually a small decline in activity between breakfast and lunch, and between lunch and dinner, the substantial loss occurring during the night. The variation in rate of secretion of saliva follows qualitatively, but not quantitatively, the variation in saccharogenic power. Variation in content of total solids also qualitatively parallels the activity of the saliva. The ingestion of coffee with breakfast does not diminish the activity of saliva collected after breakfast."

In repeating this work, particular attention was paid to the time when, and the conditions under which, the activity of salivary amylase was at its maximum.

It was found, as Walker and Shepherd have reported, that the maximum activity occurred after breakfast. The difference, however, was not always very great. The activity was seldom more than 25% greater after breakfast than before.

In order to secure a sample of salivary amylase which would have its maximum activity under normal stimulation, all samples of saliva used for this investigation were collected soon after breakfast.

Collection of Saliva.—The saliva from hospital cases was collected in 15-cc. graduated centrifuge tubes, containing 2 cc. of toluene. At least 2 cc. of saliva was collected from each subject. The tube was stoppered and was allowed to set at room temperature until it could be assayed. The amount of saliva in the centrifuge tube was diluted with a sufficient amount of water to yield a 2% solution of unfiltered saliva. It was thoroughly mixed by inversion, and was centrifuged in 15-cc. lots at 1500 revolutions per minute for five minutes. Two cubic centimeters of the clear supernatant liquid was used in the essay.

Method of Assay.—Two cubic centimeters of the clear supernatant liquid, made as explained under the collection of saliva, was added to a mixture consisting of 5 cc. of a 0.5% solution of potato starch, 1 cc. of physiological saline solution, and 2 cc. of phosphate buffer with a *p*H of 6.8, in a 6-inch Pyrex test tube. The contents were mixed by inversion, and were placed immediately in a water bath held at 40° ± 1°.

Standardized droppers were used to obtain the same amount of material in each drop. One drop of the digestion mixture was added to 1 cc. of a diluted iodine test solution (1 cc. of 0.1*N* iodine, diluted to 125 cc.). A color that was no darker than the special diluted iodine test solution was used as the test for the end point of digestion.

The Keeping Qualities of Salivary Amylase, in a Sample of Saliva, with and without Toluene

Control.—A sample of saliva, assayed fifteen minutes after it was collected, required two minutes for digestion time. One hour later it required four minutes. After the sample had been stoppered and stored at room temperature for one week, without preservatives, it required seven minutes to digest the same amount of starch.

Toluene as the Preservative.—A sample of saliva collected in a 15-cc. centrifuge tube, containing 2 cc. of toluene, and assayed within an hour, required four minutes to digest the starch. After standing at room temperature for as long as thirty-four days, it required four and one-half minutes. This test confirms the belief held by Sumner and Somers (2) that toluene can be used successfully as a preservative for enzyme preparations. They state: "Enzymes in the dry form are sometimes very stable. When in solution most enzymes do not maintain their activity very long, unless kept at low temperature. Work with enzymes lasting several hours requires the addition of some antiseptic, otherwise the enzyme may be destroyed by microorganisms, or else the results of the test may be obscured by the action of microorganisms on the substrate. Toluene is the best antiseptic; but even toluene may have a slight unfavorable effect upon the enzymes, especially if the latter are present in highly purified condition."

It is surprising that salivary amylase in saliva retains its activity for relatively long periods of time, even in the absence of antiseptics, when it is stored at room temperature. It was observed, however, that salivary amylase in saliva is much more

active when first collected than it is one hour later. This loss of activity cannot be prevented by the use of preservatives. After one hour it seems to reach a point at which it is relatively stable for many hours.

Even though the activity of salivary amylase in saliva appears not to vary in degree in the absence of preservatives for a period of one day, still toluene was used as an added safeguard, since some of the samples could not be assayed for several days. Toluene has been used as a preservative for other enzyme preparations; and since it does not show an effect upon the activity of salivary amylase and is known to prevent the destruction of enzymes by microorganisms, it was used as a preservative for all the samples of saliva collected in the hospitals.

The preservative action of toluene on salivary amylase may be seen in Table I. Eight samples of saliva were collected from eight different subjects and were allowed to stand for thirty-four days in stoppered flasks at room temperature. Each sample contained 2 cc. of toluene for every 100 cc. of 2% aqueous solution of saliva.

The Activity of Salivary Amylase Stored at Different Temperatures for Different Periods of Time.—The activity of salivary amylase in saliva is greatest when it is assayed immediately after it is collected. After standing at room temperature for only one hour it loses about half of its activity, then no further loss is observed for a period of many hours. However, if no preservative is added to the saliva a noticeable loss in activity is observed in a period of one week. It seems that saliva contains a labile activator that soon loses its effectiveness in a period of one hour at room temperature.

The storage of saliva at 50° for one hour shows no effect on the activity of salivary amylase but when the saliva is stored at 60° for one hour the salivary amylase is completely destroyed.

When saliva is stored at 2° for a period of one week the activity of salivary amylase is greater than when the saliva is first placed in storage. The reason for this increased activity has not yet been fully determined. The effect of low temperatures on such enzymes as salivary amylase is being further investigated and will be reported in a subsequent paper. The effect of different temperatures for different periods of time is summarized in Table II.

Action of Salivary Amylase in Persons with Skin Eruptions, Compared with the Action of Salivary Amylase in Persons without Skin Eruptions.—One hundred and fifty subjects without skin eruptions showed individual variations in the activity of the salivary amylase, with digestion times varying from less than one minute to six minutes. In these assays, the salivary amylase was activated with sodium chloride. When the relative stability of salivary amylase in saliva preserved under toluene was discovered, it seemed possible to use for analysis, samples of saliva from hospital cases that could not be assayed the same day the saliva was collected.

TABLE I.—THE SALIVARY AMYLASE ACTIVITY OF DIFFERENT SAMPLES OF SALIVA PRESERVED WITH TOLUENE

No. of Sample	Digestion Time after 1 Hr.	Digestion Time after 34 Days
1	2 min.	2½ min.
2	1½ min.	2 min.
3	1 min.	1½ min.
4	3 min.	3 min.
5	3½ min.	3½ min.
6	3 min.	3½ min.
7	3 min.	3 min.
8	3 min.	4 min.

Seventy-nine subjects with skin eruptions, including children and adults of both sexes, also showed individual variations. The fastest time recorded in this classification was one minute, a time which was faster than that of many subjects without skin eruptions. The slowest time, however was much slower here than in the normal subjects. One sample required more than an hour, and under no conditions of testing did this subject have a faster reacting time than forty-five minutes. This subject was classified as having acne.

If we consider those cases in which the digestion time was six minutes or less to represent normal salivary amylase activity, and those requiring more than six minutes to be subnormal, then thirty-four of the seventy-nine special cases showed normal salivary amylase activity, and forty-five would be classified as having a subnormal activity.

In this stage of our study, no definite correlation appears between the type of skin eruption and the activity of the salivary amylase. However, it is of interest that five of the seventy-nine hospital cases were classified as having acne, and that only one of these five had a normal salivary amylase activity, whereas the other four showed a subnormal activity.

SUMMARY

1. The activity of salivary amylase can be expressed in terms of digestion time.

2. The activity of salivary amylase is not

TABLE II.—THE DIGESTION TIME OF SALIVARY AMYLASE AFTER STORAGE FOR DIFFERENT PERIODS OF TIME AT VARIOUS TEMPERATURES WITH AND WITHOUT PRESERVATIVES

	Samples of Saliva			
	1 (25 samples)	2 (25 samples)	3 (3 samples)	4 (3 samples)
R. t. without preservative after 15 min.	2 min.	...	6 min.	...
R. t. without preservative after 1 hour	4 min.	3 min.	10 min.	6 min.
R. t. with preservative after 1 hour	4 min.	...	...	...
R. t. without preservative after 1 week	7 min.	7 min.	...	...
R. t. with toluene after 1 week	4 min.	...	...	...
50°, after 1 hour	4 min.	...	...	...
60°, after 1 hour	No salivary amylase	...	...	...
2°, without preservative after 1 week	.....	...	6 min.	4 min.

the same in all persons; indeed, the variation may be considerable. Although it varies in the same person from day to day, this variation is not great.

3. Salivary amylase shows a greater degree of activity when the saliva is collected after eating than at any other time.

4. Saliva contains a labile activator of salivary amylase that loses its effectiveness within one hour. This activator is not protected with toluene.

5. The activity of salivary amylase in saliva stored at room temperature, without preservative, is about the same after twenty-four hours as it is after one hour.

6. Storage of saliva at 2° for one week has an activating effect on the salivary amylase.

7. Storage of saliva at 50° for one hour has no appreciable effect on the activity of salivary amylase.

8. Saliva stored at 60° for one hour shows no salivary amylase activity.

9. Toluene is a satisfactory preservative of salivary amylase, when the saliva is to be stored for as long as one month.

10. The digestion time of salivary amylase in 150 subjects without skin eruptions was found to vary from one to six minutes.

11. The digestion time of salivary amylase in seventy-nine subjects with skin eruptions was found to vary from one minute to over one hour. Forty-five subjects had a digestion time of over six minutes, and it seems possible that people with skin eruptions may have a sluggish salivary amylase activity.

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A Preliminary Report on the Extraction of Strophanthus*

By C. LEE HUYCK†

Two tinctures were prepared, one by the N. F. VIII method and the other using 65 per cent alcohol as menstruum. Both were found to be above the N. F. standard when tested by the Knudson-Dresbach colorimetric method. The tincture extracted with 65 per cent alcohol was found to be stronger than the tincture prepared by the official method The latter remained clear for 6 months while the tincture containing more water precipitated slightly after six months at room temperature.

PRESENT-DAY accepted authorities on pharmacy (1, 2) state that it would be better to extract strophanthus with 65 per cent alcohol than with 95 per cent alcohol directed by the National Formulary (3) in the preparation of strophanthus tincture. A comparison of tinctures of strophanthus prepared with these two menstrua is given here.

Chace (4) found that strophanthus required at least seven re-percolations alternated by several periods of maceration of eight hours each for complete extraction in preparing the official tincture. Goodall (5) found that strophanthus tincture, U. S. P., retained its full activity for three years while strophanthus tincture, B. P., was inactive after two years. Tinctures containing diluted alcohol were prone to precipitate and deteriorate on standing. Haskell and Doeppers (6) found that an aqueous solution of ouabain in an amber-colored, cork-stoppered bottle suffered little or no change in strength in twenty-eight months while an aqueous solution in flint bottles lost considerable strength in twenty-two months.

Helch (7) prepared a series of tinctures by maceration with dilute and concentrated alcohols. Dilute alcohol was found to yield a tincture containing the largest amount of strophanthin. Levy and Cullen (8) found that when aqueous solution of strophanthin was stored in ampul glass the increase in alkalinity was sufficient to render the strophanthin inert. Pomeroy and Heyl (9) observed that dilute aqueous galenicals containing the mixed strophanthins deteriorated slowly. Knudson and Dresbach (10) found that the chemical colorimetric method gives an index of the potential cardiac activity of the drug. Rowe (11) found the Knudson and Dresbach colorimetric method was satisfactory only for preliminary

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assays. Haag and Hatcher (12) found that aqueous solutions of strophanthin, ouabain, and other digitalis principles kept in ampuls of soft glass deteriorated rapidly while ouabain solution in hard glass decomposed slowly. Stasiak (13) produced as good a tincture by maceration as by percolation. Rumenti (14) found that equally satisfactory results were obtained by maceration as by percolation.

Powers (15) stated that strophanthus tincture is not extensively used at the present time while Bott (16) feels that the present National Formulary method of assaying strophanthus tincture is inadequate and controversial.

EXPERIMENTAL

Strophanthus tinctures were prepared from moderately coarse strophanthus[1] powder labeled 72% received September 21, 1945. The drug was stored in sealed pasteboard containers at room temperature. Tinctures were prepared with alcohol and 65% alcohol by the official process, by the official maceration process, and by double percolation using a correspondingly larger amount of powdered strophanthus to make up for the low potency drug, namely, 69.5 Gm. of drug for 500 cc. tincture. Four hundred and seventy-five cc. was collected in each case instead of 500 cc. as directed for assayed tinctures. In double percolation the tincture was prepared by the official procedure and then the official process was repeated using the marc as drug and the tincture as menstruum. Two hundred and fifty cc. of petroleum ether was used for defatting 69.5 Gm. of drug and when percolation was used, 65 cc. of menstruum was used for moistening each portion of the drug.

The tinctures were assayed directly after preparation and after 6 months storage in flint bottles at room temperature by the Knudson-Dresbach colorimetric method (10). The method is as follows: Two cc. of strophanthus tincture is diluted with water to about 15 cc. in a 25-cc. volumetric flask. After adding 2.5 cc. of 10% lead acetate solution the contents are mixed, diluted to 25 cc. with water, shaken and filtered. In another 25-cc. volumetric flask 12.5 cc. of filtrate are treated with 1.25 cc. of 10% sodium phosphate solution to precipitate the excess lead and the contents made up to 25 cc. with water. After thorough mixing, this solution is filtered giving a clear, slightly yellow filtrate. Five cc. of this filtrate and 5 cc. of alkaline picrate solution (4.75 cc. 1% picric acid and 0.25 cc. of 10% sodium hydroxide) are mixed and the color compared with a 0.344% aqueous solution of potassium dichromate. The depth of the standard in mm. divided by the depth of the unknown is multiplied by 0.266 mg. to obtain the number of milligrams of strophanthin in terms of ouabain in the aliquot taken. The results of the analysis of the tinctures are given in Table I.

The Knudson-Dresbach colorimetric method (10) of assay is claimed to give results which are comparable to the Hatcher-Brody cat method of assay. Since the amount of drug used to make these tinctures is based on the official frog method of assay each cc. should be equal to about 5 mg. of ouabain when tested on the frog. The Knudson-Dresbach colorimetric method (10) of assay therefore gives results which are much higher than the present official assay method based on the frog. Maceration and 65% alcohol appear to give slightly higher results than percolation and 95% alcohol, but deterioration was greater in the tincture containing the lower percentage of alcohol as evidenced by the low ouabain equivalent and the formation of a precipitate. Flint glass is detrimental to the stability of strophanthus tincture. The deterioration is apparently caused by light and hydrolysis and not by a shift of the hydrogen-ion concentration.

Another lot of powdered strophanthus[2] was purchased January 19, 1947. Its strength was 76.4%. Tinctures were prepared by the official process and by maceration using a correspondingly larger amount of drug to make up for the low potency, namely 65.5 Gm. for 500 cc. of tincture, but collecting 475 cc. as directed for assayed tinctures.

These tinctures were assayed by the Knudson-Dresbach colorimetric method and the results are reported in Table II.

TABLE I.—OUABAIN EQUIVALENT OF TINCTURES OF STROPHANTHUS BY THE KNUDSON-DRESBACH COLORIMETRIC METHOD

No.	Method	Av. of Two Analyses in Mg./Cc.	pH
1	N. F. VIII (percolation with 95% alcohol)	9.3	6.3
	After 6 mo. storage	3.68	6.4
2	Percolation with 65% alcohol	10.0	6.0
	After 6 mo. storage[a]	1.67	6.2
3	Maceration with 95% alcohol	10.0	6.3
4	Maceration with 65% alcohol	11.65	5.9
5	Double percolation with 95% alcohol	9.9	6.5
6	Double percolation with 65% alcohol	10.8	6.1

[a] A definite precipitate was noted on bottom of the bottle which was not distributed by shaking.

TABLE II.—OUABAIN EQUIVALENT OF STROPHANTHUS TINCTURES BY KNUDSON-DRESBACH COLORIMETRIC METHOD

No.	Method	Av. of Two Analyses in Mg./Cc.	pH
7	N. F. VIII (percolation with 95% alcohol)	9.3	6.5
8	Percolation with 65% cohol	10.75	6.0
9	Maceration with 95% alcohol	10.3	6.3
10	Maceration with 65% alcohol	10.5	6.2

[1] S. B. Penick Co., 50 Church St., New York 7, N. Y.

[2] Lot E 1828 purchased from S. B. Penick Co., 50 Church St., New York 7, N. Y.

On the basis of the Knudson-Dresbach colorimetric assay method (10), percolation and maceration appear about equally efficient in extracting strophanthus. Sixty-five per cent alcohol appears to be a little more efficient than 95% alcohol prescribed by N. F. VIII.

Some difficulty was encountered with the Knudson-Dresbach colorimetric method (10). In spite of the fact that fresh lead acetate solution was prepared every twenty-four hours, precipitation sometimes occurred when the unknown was mixed with sodium picrate solution. Munch (17) states that if the test solution contains more than 4% alcohol, the color is too dark and erroneous results are obtained. Also, an excess alkali influences the color and produces inaccurate results. Although the colorimetric method calls for a 10% anhydrous sodium phosphate solution a consideration of the reaction involved between lead acetate and sodium phosphate shows that 10% sodium phosphate with twelve molecules of water was originally intended, and twice as much sodium phosphate as is required to react with the excess of lead acetate is directed.

The method of assay was modified as follows: Two cc. of strophanthus tincture is diluted with water to about 15 cc. in a 25-cc. volumetric flask. After adding 2.5 cc. of 10% lead acetate [$Pb(C_2H_3O_2)3H_2O$] solution, the contents are mixed, diluted to 25 cc. with distilled water, shaken and filtered. In another 25-cc. volumetric flask 12.5 cc. of filtrate are treated with 0.75 cc. 10% crystalline sodium phosphate ($Na_2HPO_4 \cdot 12H_2O$) to precipitate the excess of lead and the contents are made up to 25 cc. with water. After thorough mixing, this solution is filtered giving a clear slightly yellow filtrate. Five cc. of this filtrate and 5 cc. of alkaline picrate solution (4.75 cc. 1% picric acid and 0.25 cc. of 10% sodium hydroxide) are mixed and the color is compared with 0.344% aqueous solution of potassium dichromate. The depth of the standard in mm. divided by the depth of the unknown is multiplied by 0.266 mg. to obtain the number of milligrams of strophanthin in terms of ouabain in the aliquot taken. The results of the modified Knudson-Dresbach method are reported in Table III.

The modified Knudson-Dresbach colorimetric method (10) of assay avoids the error introduced by the use of an excessive amount of alkali and eliminates precipitation.

TABLE III.—OUABAIN EQUIVALENT OF TINCTURE OF STROPHANTHUS BY MODIFIED KNUDSON-DRESBACH COLORIMETRIC METHOD

No.	Method of Mfr.	Av. of Two Analyses in Mg./Cc.
1	N. F. VIII (percolation with 95% alcohol)	8.85
	After 6 mo. storage	2.64
2	Percolation with 65% alcohol	10.9
	After 6 mo. storage	1.57
3	Maceration with 95% alcohol	8.85
4	Maceration with 65% alcohol	10.9
5	Double percolation with 95% alcohol	9.45
6	Double percolation with 65% alcohol	8.85
7	N. F. VIII percolation with 95% alcohol	8.25
8	Percolation with 65% alcohol	10.75
9	Maceration with 95% alcohol	8.22
10	Maceration with 65% alcohol	10.22

DISCUSSION

From the results of the Knudson-Dresbach colorimetric method (10) and the modified Knudson-Dresbach colorimetric method of assay we agree with Helch (7), Pomeroy and Heyl (9), Stasiak (13), and Rumenti (14) that maceration and dilute alcohol produce tinctures more potent than percolation with 95% alcohol. However, tinctures containing diluted alcohol are prone to precipitate and deteriorate on standing as recorded by Goodall (5). The difficulty in extracting strophanthus recorded by Chace (4) was not experienced. Haskell and Doeppers (6), Haag and Hatcher (12), and Levy and Cullen (8) concluded that flint and ampul glass cause rapid deterioration of the tincture. Rowe (11) noted that the results of the picric acid colorimetric method of assay of Knudson and Dresbach (10) did not agree with the results of the Houghton frog method and concluded that the colorimetric method is only satisfactory for preliminary assays.

SUMMARY

Judging from the colorimetric assay of Knudson and Dresbach (10) and a more accurate modification of the colorimetric method: (*a*) Strophanthus is extracted satisfactorily by either percolation or maceration. (*b*) Although 65% alcohol extracts strophanthus a little more efficiently than alcohol U. S. P., the tincture containing the lower percentage of alcohol is not as stable as the tincture prepared with alcohol U. S. P. (*c*) Strophanthus tincture deteriorates rapidly when stored in flint glass. (*d*) The colorimetric assay method of Knudson and Dresbach (10) and a more accurate modified colorimetric method give results which are higher than the official frog assay method.

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A Preliminary Survey of the Occurrence of Nordihydroguaiaretic Acid in *Larrea divaricata**

By OLE GISVOLD†

A method has been developed for the isolation of crystalline nordihydroguaiaretic acid from *Larrea divaricata*. This method has been applied quantitatively to specimens of this plant collected from California, Arizona, Texas, and Argentina. The results show that crystalline nordihydroguaiaretic acid was isolated from the surface of the plant in amounts equal to 2 to 6.5 per cent. One specimen that was obtained from Texas yielded no nordihydroguaiaretic acid even though much phenolic material was present.

IT HAS BEEN shown that most, if not all, of the phenolic materials of *Larrea divaricata* Fam. *Zygophyllaceae*, a perennial evergreen shrub, are present as a resinous exudate on the surface of the leaves and stems (1). It has also been shown that the nordihydroguaiaretic acid content of this plant is present in this resin. Nordihydroguaiaretic acid is an excellent antioxidant (2), antiseptic and is relatively nontoxic. It is being produced on a commercial scale. This production is made possible by the vast areas of the Southwest U. S., Mexico, and South America where *L. divaricata* grows on well-drained upland soil that is not suitable for cultivation. This commercial production was also made possible by the development of practical methods for the isolation of nordihydroguaiaretic acid.

It is well known that the time of the year, climatic, and soil conditions play a more or less pronounced role in the amounts and composition of certain constituents produced by plants. The amounts of nordihydroguaiaretic acid produced by *Larrea divaricata* under the above conditions would have both scientific and practical significance. One difficulty in connection with such an investigation lay in the collection of the plant. The samples investigated and reported in this paper are somewhat at random due to the abovementioned difficulty. A second difficulty dealt with quantitative procedure for the determination of the amount of N. D. G. A. present. The method adopted and reported in this paper was the culmination of a large number of attempts to develop a gravimetric procedure for the determination of crystalline N. D. G. A. No claim is made for the efficiency or accuracy of the procedure because losses of N. D. G. A. are quite unavoidable in a number of the steps. Nevertheless, it has furnished a guide up to the present to measure the amount of isolable crystalline N. D. G. A. by these techniques which can be duplicated on a large scale.

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EXPERIMENTAL

The plant samples used in these investigations consisted of, in most cases, small twigs, leaves, and flowering tops that were, when air dried, ground to a very coarse powder in a Jacobson laboratory mill. In several cases, samples of leaves and their petioles only were sent by the collector. The large twigs and stems were not included because previous investigations had shown that only small amounts of resin and N. D. G. A. could be obtained from them. Therefore, from a practical standpoint, they would have no value in so far as their N. D. G. A. content is concerned.

The samples of *Larrea divaricata* were examined for their N. D. G. A. content as follows: 200-Gm. samples were percolated rapidly (not over an hour) with about 800 cc. of 5% aqueous solution of sodium hydroxide containing 2.5% sodium hydrosulfite. The deep red percolate was acidulated with dilute hydrochloric acid. Because the extraction had to be carried out quite rapidly in order to avoid too much destruction of the N. D. G. A. in the presence of alkali, a variable was introduced which could lead to incomplete extraction. If larger quantities of solvent were used, the dilutions became so great that greater error would be obtained as discussed in the subsequent acidification. The mixture was stirred very rapidly during acidification in an attempt to prevent the occlusion of alkali and partial neutralization of the semisolid dark brown precipitate that formed immediately. The mixture was allowed to stand several days in order to obtain as complete precipitation as possible. In most instances, the precipitate still contained some sodium salts of the phenolic substances. In many cases, the supernatant yellow mother liquors contained some colloidal phenolic substances that upon examination were shown to yield negligible to small quantities of N. D. G. A. Modifications of the techniques of acidification did not result in any improvement and no uniformity was obtained. This, therefore, became one of the steps in which some N. D. G. A. could be lost. The mother liquor was decanted from the semisolid precipitate and covered with about 150 cc. of isopropyl ether. A few cc. of

30% hypophosphorus acid were added to neutralize any excess alkali that might be present. The mixture was heated to boiling and stirred very vigorously with a mechanical stirrer for about 5 to 10 minutes. The ether was decanted and the residue extracted with 2 additional 150-cc. portions each of hot isopropyl ether. An ether-insoluble residue was obtained and this varied in amount depending upon the sample examined. Likewise, the amount of isopropyl ether-soluble material varied. Some of the samples analyzed were examined for the amounts of phenolics that could be adsorbed on sodium carbonate. Where this was done the following procedure was employed.

The isopropyl ether extracts were combined, filtered, and about 60 Gm. of powdered sodium carbonate monohydrate in 15-Gm. portions were added while the mixture was stirred vigorously. The isopropyl ether was decanted and the sodium carbonate adsorbate washed with several 50-cc. portions of isopropyl ether. The combined ether solutions were filtered, treated with several drops of 30% hypophosphorus acid. The ether solution was concentrated on the steam bath under a vacuum until the mixture assumed the consistency of a thin syrup. Ten to 20 cc. of ethylene dichloride was added and the mixture seeded with N. D. G. A. and the whole allowed to stand overnight in order to insure maximum crystallization. The mixture became a semisolid crystalline mass. It was then diluted with about 15 to 25 cc. of warm ethylene dichloride and the crystals of N. D. G. A. were collected in a sintered glass funnel. The crystalline N. D. G. A. was washed with several portions of warm ethylene dichloride and dried to constant weight.

The ethylene dichloride soluble portion and washings were combined and the solvent removed by heating them on a tared Pyrex evaporating dish. Difficulty was encountered in removing the last traces of ethylene dichloride, and heating in an oven at 70° to 80° was necessary to obtain the desired results.

The sodium carbonate adsorbate was acidified with dilute hydrochloric acid and the insoluble phenolic fraction collected, washed with water, and dried to constant weight in an oven at 80°.

The following table contains the results obtained when the samples of *Larrea divaricata* were examined by the procedures described above. Because 200-Gm. samples were used, the results were divided by two so that they could be expressed as weight percentages.

TABLE I.—EXTRACTION DATA ON *Larrea divaricata*

Source	% Isopropyl Ether Insoluble Portion	% Ethylene Dichloride Soluble Fraction	% Sodium Carbonate Adsorbate	% N. D. G. A.
Calif. 1	3.25	5.65	..	5.00
Calif. 2	3.25	6.40	..	6.45
Calif. 3	3.40	4.45	2.10	6.00
Calif. 3	3.75	4.00	1.60	6.55
Calif. 4	2.50	3.70	2.10	5.60
Calif. 4	1.50	3.75	2.15	5.25
Calif. 5	2.00	3.30	2.40	3.95
Calif. 5	1.80	3.60	2.00	4.05
Calif. 6	1.85	1.70	1.90	4.05
Calif. 6	2.20	2.25	1.50	3.85
Calif. 7	2.50	5.30	2.20	3.40
Calif. 7	2.00	4.50	2.80	3.30
Texas 8	5.75	5.75	1.87	none
Texas 8	6.00	5.50	1.60	none
Texas 9	5.10	3.10	1.00	2.50
Texas 9	4.75	3.00	1.40	2.75
Arizona 10	4.00	5.65	..	4.85
Arizona 10	3.80	5.75	..	4.50
Arizona 11	4.50	6.30	..	5.20
Arizona 11	3.85	4.95	..	5.25
Arizona 12	2.30	5.60	..	3.50
Arizona 12	2.10	5.00	..	3.15
Arizona 12	2.80	5.45	..	3.60
Arizona 13	3.20	3.10	0.65	2.00
Arizona 13	2.50	3.45	1.15	2.50
Argentina 14	4.75	4.15	..	2.25
Argentina 14	4.00	4.50	..	2.50

Key to table.—Samples obtained from California numbered 1 to 6 were very kindly supplied by Dr. Robert Emerson of the California Institute of Technology. California 1 was collected on August 27, 1944, near Highway 6 in the Indian Wells Valley about two miles north of Indian Wells service station, altitude 1500 feet, rainfall approximately 4 inches annually. Numbers 2 and 3 were collected on November 16, 1944, and April 12, 1945, respectively, from the same area. Number 4 was collected June 13, 1945, from the Mojave Desert between Lancaster and Palmdale, which has an altitude of 2000 feet. Number 5 was collected June 13, 1945, in the Inyo-Kern Valley near Indian Wells at an altitude somewhat higher than Indian Wells. Number 6 was collected in early June in the Owens Valley above the Haiwee Reservoir at an altitude of 4000 feet. Sample No. 7 was obtained from a well-watered garden in California. Samples 8 and 9 were ob-

tained through the courtesy of the Wm. J. Stange Co. of Chicago and were collected near Presido, Texas. Samples 10 and 11 consisted of leaves and petioles only and were collected near Topock, Arizona, by John Booth. No. 10 was obtained from a "dry" area and No. 11 from a "wet" area. No. 12 was also obtained from Mr. Booth. No. 13 was supplied by Mr. A. A. Burden of Scottsdale, Arizona, and was collected on April 17, 1946, from an area near the base of a mountain that had an altitude of 1600 feet and an average rainfall of 7 inches. No. 14 was obtained from Argentina through the courtesy of Dr. Horacio R. Descale, Director of the Institute Miguel Lillo, Tucumann, Argentina.

SUMMARY AND CONCLUSIONS

Disregarding the inaccuracies inherent in the method employed, sufficient significant data were obtained in the assays of the various specimens of *Larrea divaricata* collected from a number of habitats. The specimens obtained from California yielded about 4 per cent crystalline N. D. G. A. from the highest altitude, about 4000 ft., to about 6 per cent N. D. G. A. from the lowest altitude, about 1500 ft. The time of the year did not make too much significant difference in the amounts of N. D. G. A. in the specimens collected from California at the lowest altitude, i.e., 1500 feet. No such comparison was possible with the remainder of the specimens because of the difficulty of obtaining samples at different seasons of the year.

It is interesting to note that about 5 per cent N. D. G. A. was obtained from the leaves collected in Arizona whereas about 3.5 per cent N. D. G. A. was found when the small twigs were also included. One sample from Arizona yielded only 2.25% N. D. G. A.

Some specimens from Texas yielded no N. D. G. A. even though considerable amounts of phenolic substances were present as an exudate. Other specimens from Texas gave low yields of N. D. G. A., i.e., about 2.6 per cent.

It is interesting to note that *Larrea divaricata* grows in Argentina and that it does produce N. D. G. A.

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The Growth Effects Produced by 2,4-Dichlorophenoxyacetic Acid Applied to Stems of *Datura Stramonium* L.*

By H. W. YOUNGKEN, JR.

Varying concentrations of 2,4-dichlorophenoxyacetic acid were applied in lanolin and petrolatum bases to the stem portions of *Datura stramonium* and *Datura tatula*. Marked changes occurred in cellular proliferations, stem growth, leaf pubescence, texture, and flower and fruit development following the use of concentrations of from 0.1 per cent to 5.0 per cent. Plants appeared to show little to no toxic effects when treated with lower concentrations. Those seedlings treated with 0.001 per cent and 0.0001 per cent of the chemical showed as vigorous a growth response as the controls.

SINCE the studies of Zimmerman and Hitchcock (1–3), Koepfli and Thimann (4) and others on the responses shown by plants to treatments with various chlorophenoxyacetic acid compounds, the systemic and localized growth-inhibiting effects of these compounds have been made use of in many ways. Principally, 2,4-dichlorophenoxyacetic acid has been employed in a spray or solution as a general herbicide. Zimmerman and Hitchcock (1–3) induced seedless tomatoes and cucumbers with concentrations of 2,4-dichlorophenoxyacetic acid from 5 to 11 p. p. m. in water and 10 to 25 p. p. m. in carbowax. These workers demonstrated further that chlorophenoxyacetic acid compounds including 2,4-dichlorophenoxyacetic acid move up or down the stem of tomato plants, tobacco, etc. When applied in aqueous media to soil, or when sprayed on leaves, or when applied in lanolin base to stem regions systemic responses such as leaf

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epinasty, curvature, and marked cellular proliferations were produced.

Hamner, Tukey, Kraus, Mitchell, and others (3,5) established the fact that phenoxyacetic acids of several types produce a herbicidal action which could be utilized for many of the wide-leaf weed plants. The most selective herbicidal action has resulted with chlorine introduced into the *ortho* and *para* positions of the phenoxyacetic acid ring structure.

The minimum concentration of 2,4-dichlorophenoxyacetic acid for producing formative effects in plants has not been fully determined, but is estimated by Zimmerman (1) to be in the neighborhood of one part of chemical to 50 million parts of solvent when used in solution; or from 0.0003 per cent to 0.0015 per cent in lanolin. Koepfli (4) has estimated that the lowest limit for inducing minimum responses of 2,4-dichlorophenoxyacetic acid in a lanolin base is near 0.015 mg./Gm.

On the basis of the established systemic growth responses following the application of 2,4-dichlorophenoxyacetic acid to certain vegetable and broad-leaf weed plants, it appeared of interest to determine what effect this substance would have upon seedlings, growing plants, and the alkaloid yield of *Datura stramonium* when applied to stem portions.

MATERIALS AND METHODS

For the first phase of the experiment during which growth response in young seedlings of *Datura* plants was observed, seedlings were transplanted to experimental pots when approximately 3 inches in height and while cotyledonary leaves were present. Flower buds had not appeared. Sufficient time was allowed for each transplanting to fully recover.

For the second phase of the problem older plants of *Datura stramonium* were selected. These were allowed to reach a height of approximately 8–10 inches before treatment was begun. Each plant had developed a single opened flower in the axillary region of the first forked branching of the stem. One group of control plants was set aside for each stage of treatment.

Chemically pure 2,4-dichlorophenoxyacetic acid obtained from Eastman Kodak Company was incorporated into hydrous lanolin (U. S. P.) to make inoculation ointments of the following strengths: 50 mg./Gm. (5%), 25 mg./Gm. (2.5%), 10 mg./Gm. (1%), 1 mg./Gm. (0.1%), 0.1 mg./Gm. (0.01%), 0.01 mg./Gm. (0.001%), and 0.001 mg./Gm. (0.0001%). These ointments were applied by means of a cleaned glass rod to the lower stem of each plant, approximately 1 inch from the stem base and above soil level. A strip of ointment about $^3/_4$ inch in length and $^1/_4$ inch wide was applied using a vertical movement. Efforts were made to apply all concentrations in a uniform manner. Alkaloid assays were made using the methods for total alkaloids of stramonium as described in U. S. P. XIII (6).

Seedlings and maturing *Datura* plants were grown in a soil consisting of two parts humus, including leaf mold, and one portion fresh water sand.

EXPERIMENTAL

Seedling Growth Response.—An assumption is made that the actively growing meristematic tissues of seedlings should show most sensitivity to an induced foreign chemical. Therefore, it was felt that by measuring the action of various concentrated and dilute 2,4-dichlorophenoxyacetic acid ointments when applied to the stems of *Datura stramonium* seedlings some effect might be derived upon which to base future stem treatments of older growing *Datura* plants. Conversely, the possibility might exist whereby concentrations, while toxic to seedlings, might be resisted by older plants. This would be advantageous for alkaloid studies. The stems of seedlings were treated with application of 5%, 2.5%, 1%, 0.1%, 0.01%, 0.001%, and 0.0001% when approximately 3 inches in height and prior to the formation of flower buds. For these treatments four seedlings were treated with each concentration.

Seedlings treated with 2,4-dichlorophenoxyacetic acid ointment of 5% and 2.5% showed a growth response within twenty-four hours following the first application. Small leafy portions became dull green in appearance and showed epinasty. Thirty-six hours later these leaves became darker green and bore small warty protuberances under leaf surfaces. The stems of each plant showed marked bending. Within forty-eight hours leaves drooped beyond recovery, and developed a marked roughness on the dorsal surface. Fifteen days following the treatment, the leaves of two plants appeared to recover from the effects. Newer leaves, however, became severely curled and roughened, and the basal hypocotyls were swollen with numerous cellular proliferations. Bending progressed in two seedlings to form the letter "S"; seedlings did not recover and were dead in eight days.

Three seedlings treated with 10 mg./Gm. (1%) in lanolin at first showed effects of a similar type as did those treated with higher concentrations. However, the seedlings were obviously affected to a lesser degree. These seedlings were markedly stunted in growth but survived the treatment for about twenty days before they first lay prostrate. The fact that some new growth did appear showed that there was a slower systemic response to the full effects of such a concentration. Newly developed leaves became rough and dark green. Seven days following the application a gradual swelling developed below the site of ointment treatment on the stem. Seventeen days after the treatment this stem swelling showed numerous small, white, warty outgrowths. For a distance of from 1 to $1^1/_2$ inches above the ointment application, and on the same side, numerous small papular nodules developed. These spread around the stem but persisted in largest numbers on the side of the ointment smear. Flower buds failed to develop in any of the plants.

Twelve seedlings were treated with ointments of 0.1%, 0.01%, 0.001%, and 0.0001% in lanolin. Those on which 0.1% and 0.01% were applied showed definite abnormal growth response. Although stunted in growth all seedlings recovered sufficiently to form flowering plants. Those treated with 0.1% developed slightly roughened upper

leaves and a slight degree of stem bending. No basal stem swelling was observed although some warty outgrowths appeared above the ointment smear. These were not nearly as abundant as produced following higher concentrations. Ten days following the treatment, seedlings began to recover from the effects although newer leaves showed curving and roughness and appeared much darker green in color than those of the controls. Seedlings treated with 0.01% showed a lesser degree of the above effects although these appeared definitely to be retarded in growth. In the latter case, the effects such as leaf roughness and leaf marginal curling appeared within forty-eight hours following treatment, but after five days these plants seemed to recover gradually.

Seedlings treated with 0.001% (0.01 mg./Gm.) and 0.0001% (0.001 mg./Gm.) in lanolin applied to the basal region of the stem showed no abnormal effect of any degree. In general, these seedlings grew as vigorously as did control seedlings. Ten days following the first application of the ointment, the remnant of the first smear was wiped off and a new application was made at the same spot. Ten days following this treatment a very slight degree of leaf roughening appeared on one seedling treated with 0.001%. This persisted but newer leaves failed to show such an effect. There appeared to be no effects produced in those seedlings treated with 0.0001%. These seedlings did not show any greater stimulating response than did control seedlings. Seedlings treated with 0.01% to 0.001% were then allowed to grow to mature plants. Seed was collected and planted.

From the effects observed by treating the above seedlings with varying concentrations of 2,4-dichlorophenoxyacetic acid, it was felt that the minimum effective concentration of the chemical when applied in lanolin would be somewhere between 0.01% and 0.001%. Lowest concentrations used, 0.0001% (0.001 mg./Gm.), failed to show any stimulating effect greater than control seedlings. Seedlings showing growth responses to the chemical applied in higher concentrations appeared to be affected systemically and in proportion to the concentration used. This was evident as soon as the newer uppermost leaves formed and showed a roughness and curling.

On the basis of the results observed following treatments of seedlings with concentrations of 2,4-dichlorophenoxyacetic acid in lanolin, *Datura stramonium* plants measuring 8–10 inches in height and with basal flower opened were similarly treated. Each of six plants was treated on May 14 and June 2 with 5.0%, 2.5%, 1.0%, 0.1%, 0.01%, 0.001%, and 0.0001% 2,4-dichlorophenoxyacetic acid in lanolin. Systemic effects were observed in all plants treated with from 5% to 0.1% following the first application. These were most rapid in the higher strengths as previously seen in the seedlings. The first visible response was seen some eight days following the initial treatment. Leaf roughing, epinasty, and stem swelling appeared almost simultaneously in plants treated with 5% and 2.5%. In each case, cellular proliferations in the basal portion of the stem below the site of ointment application preceded the effects seen in the leaves. The new uppermost leaves showed responses prior to the lower more mature leaves. These leaf responses began at varying points. Usually the leaf margin curved downward, beginning at the apex, the inward curvature spreading gradually to the base until the leaf had taken the form of a tubular structure. Accompanying such an effect pubescence along the dorsal surface increased markedly. This appeared also intensely stimulated along the leaf petioles. All leaves became much darker green in color than those of the controls. Zimmerman (3) has reported a similar condition among leaves of *Nicotiana* spp. and tomato plants treated with 2,4-dichlorophenoxyacetic acid of varying concentrations and has suggested that some effect was taking place which interfered with translocation of synthesized food in the leaf.

Forty-five days following the first application of 2,4-dichlorophenoxyacetic acid to the stems of plants, the following responses were observed in plants treated with higher concentrations (5% to 1%).

Stems.—The angle of lower stem branching, which was dichotomously acute from a 10–30° angle in control plants, was from 42° to 160° in treated plants (Fig. 1). Branching of stems higher up

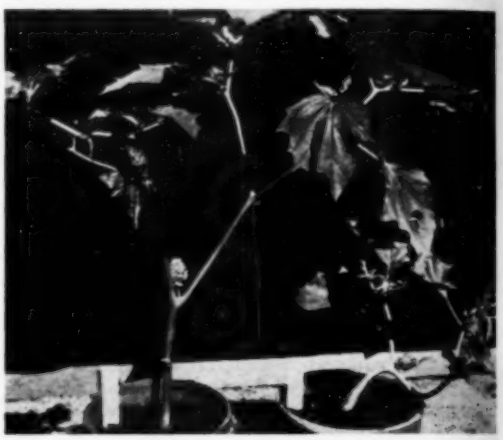

Fig. 1.—Control plant, *Datura stramonium* L., on left; right, plant treated with 5 per cent 2,4-dichlorophenoxyacetic acid applied to basal stem portion. Forty-five days following initial treatment.

showed a similar effect. Severe stem enlargement or swelling had occurred below, above, and around the ointment smear. Most treated plants measured 1⅝ inches through widest diameter. Swollen stem bases were characterized by numerous warty outgrowths of various sizes (Figs. 2 and 3). Several cross and longitudinal sections were made through stem regions immediately above and below the site of 2,4-dichlorophenoxyacetic acid ointment application in the area of widest growth and cellular proliferations. Serial sections revealed most warty outgrowths to be formed by increased localized cell activity in the outer cortex of the stem. This was not always accompanied by primordial tissues emanating from the stele as was looked for. Very few such outgrowths of primordial tissues were observed. In the latter case primordial trace

bundles appeared in clusters and pierced the cortical regions in a vermiform manner. Many of the latter trace bundles ended in the cortex. Such large warty outgrowths covered the stem periphery, and were observed higher up in plants along the petioles of affected leaves. Larger papilose eruptions appeared from the region of ointment application to about 3 inches above and along the same side of the stem. Immediately surrounding the area of chemical treatment cells became brown and fibrous. Such cells gave qualitative tests with Sudan IV and concentrated KOH for suberin. Lignification tests with phloroglucin-HCl were negative. More than one-half the diameter of some stems showed such a scar formation.

Fig. 2.—Stramonium plant four weeks following application of 5 per cent 2,4-dichlorophenoxyacetic acid to stem. Note stem widening and bending with cellular proliferations along and above scar tissue.

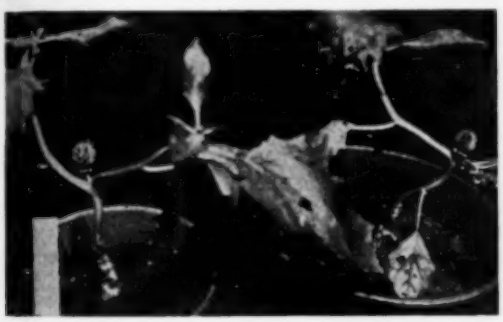

Fig. 3.—Showing marked stem bending and cellular proliferations in *Datura stramonium* plants treated with 2.5 per cent of 2,4-dichlorophenoxyacetic acid. Thirty days following application of ointment to stem.

There was marked curvature of stems above the first branching and at a wide angle (Figs. 1, 2, and 3). Younger stems appeared normal during the growth period but became obtusely divided as they matured. This stem effect was from lower stems upward.

The internodal length of treated plants measured from $2^1/_2$ inches to $3^1/_2$ inches below the first branching to a maximum of 10 inches higher in plants treated with 1%. Plants treated with 5% and 2.5% branched two to three times and between the two second nodes measured up to 5 inches. Control plants measured from 7 inches to $8^1/_2$ inches below the first branching and up to 12 inches above, and branched more profusely.

Leaves.—Varying responses were noted during leaf development. First formed leaves developed normally. No effects were noticed until about fifteen days following the first application of 2,4-dichlorophenoxyacetic acid ointment. At this time most of the terminal leaves of 5% and 2.5% concentrations appeared to show a marked roughing, curvature, and darkening. The effect increased rapidly and was accompanied by intense trichome formation. At the end of forty-five days upper leaves were folded sharply inward at the margins, becoming tubular (Figs. 4 and 5). Numerous small

Fig. 4.—Typical formative effects produced in leaves of *D. stramonium* when 2,4-dichlorophenoxyacetic acid ointment of from 0.1 per cent to 5 per cent is applied to basal stem region.

Fig. 5.—Showing spineless fruit and leaf roughness, increased pubescence and warty outgrowths produced in upper portion of plant approximately four weeks following application of 1 per cent 2,4-dichlorophenoxyacetic acid to lower stem portion.

warty outgrowths appeared on both surfaces, mostly on ventral surfaces and near veins. Pubescence also increased markedly along leaf veins and along the petioles. This response was observed to occur in lower leaves but slowly and with less intensity. Such leaves showed the effects last and only after the second application.

Flower Development.—There was a marked retarding of flower development following the first application of these higher percentages of 2,4-dichlorophenoxyacetic acid ointment. Only lower flowers reached maturity. These developed capsular fruits. Flower buds formed elsewhere but remained unopened. Carpels and stamens of these were dwarfed. Pollen was observed to form in some anthers. Generally stamens failed to develop. In three unopened flowers a marked papillose outgrowth of irregular structure formed around the ovary.

Fruits.—Most plants treated with 5% and 2.5% 2,4-dichlorophenoxyacetic acid in lanolin bore at least one mature capsular fruit. Two plants developed two mature fruits each. In general, those mature fruits formed appeared as large as those of control plants. Most became darker green in color than those of controls. Two plants which were treated with 1% to 0.1% concentration, and on which more mature fruits developed, formed spineless capsules (Fig. 5). Such capsules did not appear in any of the controls nor did they occur in several hundred parent control plants of previous generations. These bald capsules were examined three weeks later for seed. At this time seeds were chocolate-brown in color and were of two general sizes and shapes. The larger seeds were flattened kidney shaped with roughened coats and of the same appearance as those of control plants. Other seeds appeared vermiform to crescent or horn shaped and much smaller in size and narrower than the first group. Both groups were set aside for germination and F_2 studies. Other capsules developed only a few spines on the upper portion of each valve. Seeds of these capsules showed development similar to the spineless group.

Roots.—There appeared to be some effects as compared to normal growth in roots of plants treated with 5% to 1%. The outstanding macroscopic effect was the curvature and bending of that portion of the tap root about $^1/_2$ inch below the stem crown. This was observed in all plants treated with higher concentrations. Adventitious roots emerged from a position slightly above the crown and above the soil level. These grew downward into the soil. In general, the tap root appeared wider in diameter than that of the controls. Lateral roots were not as numerous as those seen in controls. The chemical applied in higher concentrations appeared to inhibit over-all root development.

Most mature plants that had been treated with 5%, 2.5%, and 1% varied in height of growth measured from stem base to tip of highest leaf in direct proportion to the strength of 2,4-dichlorophenoxyacetic acid ointment applied. Plants treated with 5% and 2.5% reached a height of from 12 to 22 inches. Plants treated with 1% ranged in height from 17 to 29 inches. Control plants reached a height of from 26 to 40 inches.

Treatments with Lower Concentrations.—There appeared to be some growth response in two plants treated with 0.1% eleven days following the second application of the 2,4-dichlorophenoxyacetic acid in lanolin. This response was characteristic of that observed in leaf and fruit development of plants treated with as much as 1% ointment. Leaf roughening, increased pubescence, and curvature at the margins began in the apex region and spread to the petioles. This was to a lesser degree than observed following higher concentrations but was observed in all but 17 out of 21 leaves. The above plant also developed two capsular fruits, which did not form spines. Other fruits formed normally. Five additional *Datura* plants treated with 0.1% failed to show these effects.

The stem branching on all plants treated with 1% ointment appeared as normal as that of the control plants. No basal stem enlargement or unusual cellular proliferations appeared.

Datura stramonium plants treated with lower concentrations of 2,4-dichlorophenoxyacetic acid in lanolin, i.e., those of 0.01%, 0.001%, and 0.0001% which were applied twice, showed no toxic responses. In general, these plants grew as vigorously as the controls. Six plants to which 0.001% ointment had been applied twice and six control plants were transplanted to a floor bed. These were allowed to recover sufficiently from transplanting. Two additional applications of the 0.001% ointment were made at fifteen-day intervals and to the same area on the stem. No growth responses were observed. Flowers and fruits formed. When compared with control plants, treated plants bore 11 to 14 mature fruits whereas the former formed 5 to 11 mature fruits. These plants, although failing to show the toxic manifestations typical for higher concentrations, developed about the same degree of stem, leaf, and flower growth as did controls. No unusual growth-stimulating effects were observed with the exception of a slight increase in numbers of fruit formed. Such was not considered to be significant.

Roots of plants treated with 0.01% to 0.0001% appeared equal in size to those of controls. No unusual bending of the tap root was observed. It appeared that 2,4-dichlorophenoxyacetic acid applied to the stem had no abnormal stimulant or inhibiting effect in the root system.

Leaves were collected from plants treated with 0.001% 2,4-dichlorophenoxyacetic ointment and from control plants forty-eight hours following a fourth application of the chemical, and when each group was eighty-five days old from date of first leaf formation, these were dried at 80° F., then powdered and assayed for total alkaloid content as of total alkaloids of Stramonium U. S. P.

Table I illustrates the comparative results.

DISCUSSION

The fact that growth effects produced by concentrations of 2,4-dichlorophenoxyacetic acid from 5% to 0.01% when applied to the stems of *Datura stramonium*, are morphogenetic in character, indicates that this species responds to the typical phenoxyacetic acid activity as described in similar treatments by Zimmerman (1) for other members of the *Solanaceae* family. It is reasonable to assume, therefore, that the esters, salts, and amides of phenoxy compounds would have a similar effect on *Datura*.

TABLE I.—*Datura stramonium:* PER CENT OF ALKALOIDS FOLLOWING TREATMENTS WITH 0.001% 2,4-DICHLOROPHENOXYACETIC ACID

Treatment Lot No.*	Wt., Gm.	% Alkaloids	Control Lot No.	Wt., Gm.	% Alkaloids
1	10	0.2840	1	10	0.4033
2	10	0.3308	2	10	0.3491
3	10	0.2785	3	10	0.2871
4	10	0.3852	4	10	0.3120
	Av.	0.3198		Av.	0.3376

* Treatment consisted of applying 10 mg./Gm. of 2,4-dichlorophenoxyacetic acid in lanolin to stem portions.

Modifications of organs were produced in seedlings and more mature plants in such a way that newer cell growth appeared to be effected first. Older growth was effected later. This indicates that this compound exerts most activity on the cells in which somatic mitoses are taking place. The fact that no unusual flower or seed development appeared in the first generation might indicate that the meiotic figures of this period were generally not severely affected. Consistent fruit setting further is indicative of this fact. It seems likely that the chemical causes increased lateral cellular growth in stems when high concentrations are used. So rapid is lateral growth that more numerous proliferations and nodule outgrowths appear. These are not always accompanied by increased pericambium activity but more often seem to be a phenomenon of the phellogen or other cells of the outer cortical parenchyma.

The darker color of the leaves following concentrations of 0.01% and above when applied to stems and the leaf monstrosities produced, such as marked surface roughening, thickening, curling, epinasty, increased pubescence and warty outgrowths, are factors indicating that *Datura* plants are affected systemically by 2,4-dichlorophenoxyacetic acid. Increase in the number of leaf hairs would further indicate marked cellular growth in the outer mesophyll and epidermis of the leaves.

The fact that there was no growth-stimulant action greater in comparison with control plants when nontoxic concentrations of 0.001% and 0.0001% of 2,4-dichlorophenoxyacetic acid in lanolin were applied to the stems is evidence that such concentrations of the chemical are either neutralized by some antagonist within the plant or that the chemical can be stored in smaller concentrations. Further studies are contemplated on F_2 and F_3 generations of *Datura* seeds collected as well as on the fate of 2,4-dichlorophenoxyacetic acid in the plant.

The application of 2,4-dichlorophenoxyacetic acid to the stem of *Datura* in all concentrations used failed to produce parthenocarpy although the systemic action of high concentrations inhibited flower and fruit development.

The difference of 0.0178% total average of alkaloids (Table I) between control plants and treated plants was not considered significant. It appears from these preliminary alkaloidal observations that 2,4-dichlorophenoxyacetic acid in nontoxic ointment treatments of 0.001% and applied to stems has no appreciable effect upon amount of total alkaloids developed in the leaves of *Datura*. Leaf portions of plants treated with higher concentrations are altered severely. It is possible that alkaloid formation in these might be altered. These investigations are being continued.

SUMMARY

Several concentrations of 2,4-dichlorophenoxyacetic acid in lanolin have been applied at intervals to the lower stem regions of *Datura stramonium* plants. Localized and systemic effects such as intensive cellular outgrowths, stem widening, and bending have been observed. Leaves showed marked thickening, roughness, curvature, increased pubescence, and epinasty following the application of concentrations of 0.1% (1.0 mg./Gm.) or above. Spineless fruits were produced consistently. New somatic cellular growth was most affected throughout each plant.

The minimum growth response was found to be in the vicinity of 0.1 per cent (0.1 mg./Gm.). Plants treated with 0.001 per cent (0.01 mg./Gm.) and below showed no abnormal macroscopic growth responses.

Total alkaloids were measured quantitatively in leaves of plants previously treated with 0.001% 2,4-dichlorophenoxyacetic acid in lanolin and applied to lower stems. There were no significant differences between total alkaloid yield in these and that of control plants.

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Microscopic Identification of Some of the Crystalline Sulfonamides*

By GEORGE L. KEENAN†

The application of the immersion method for the microscopic identification of the crystalline sulfonamides has been found very useful in determinative analysis. Optical crystallographic data, for six crystalline sulfonamides, microchemical tests, and optical constants for crystalline complexes formed with specific reagents are presented.

THE PURPOSE of this paper on sulfanilamide, sulfadiazine, sulfapyridine, sulfathiazole, sulfaguanidine, and sulfamerizine is to record the results of a comparative microscopic study of them based on microchemical tests and optical crystallographic determinations. The parallel application of both methods of attack has been found very useful in determinative analysis. Some of these data have appeared elsewhere but set apart as either microchemical tests or condensed optical crystallographic descriptions. In the following monographs for each substance, the significant optical crystallographic data are given first, followed by the diagnostic microchemical tests and frequently by the optical crystallographic description of a crystalline complex formed with a specific reagent. In accordance with this plan, the descriptions of these substances are as follows.

Sulfanilamide

Optical Crystallographic Data.—Sulfanilamide consists of a colorless, crystalline powder, the fragments lacking in distinctive habit. The significant refractive indices are $\alpha = 1.570$, $n_i = 1.677$, $\gamma = >1.733$. Interference figures are not shown in convergent polarized light (crossed nicols), therefore the β-value could not be determined with certainty. However, for determinative purposes, the value, $n_i = 1.677$ occurs very frequently and on this account is very useful for a rapid and accurate identification (1).

Microchemical Identification.—Yakowitz (2) has already called attention to the reaction produced by sulfanilamide with the aromatic aldehydes, especially benzaldehyde and cinnamon oil. The plates formed consist of parallelograms and are quite significant.

Sulfanilamide also forms a crystalline complex with finely powdered silver nitrate. The test is best carried out by mounting a small portion of the test material in a drop of 50% alcohol on an object slide and then dropping into the preparation some of the silver nitrate powder. Immediately rectangular plates form, frequently in circular aggregates and with notched ends. These are allowed to dry on the object slide at room temperature and set aside for subsequent examination with the polarizing microscope.

Optical Crystallographic Properties of Crystalline Complex Formed with Powdered Silver Nitrate.—With crossed nicols, the crystalline precipitate described above exhibits anomalous interference colors, consisting of peculiar shades of blue and purple. The rectangular plates show parallel extinction, those with maximum double refraction giving the following significant refractive indices: $\alpha = 1.590$ (very commonly shown lengthwise on elongated plates), and $\gamma = 1.734$ (commonly shown crosswise on plates); both ± 0.003. The significant microscopic features, therefore, of this crystalline complex are the habit of the crystals, the peculiar interference colors shown with crossed nicols, and the refractive indices shown in the respective orientations.

Sulfadiazine

Optical Crystallographic Data.—Sulfadiazine consists of very small colorless rods when examined microscopically in ordinary light. These show parallel extinction with crossed nicols and the sign of elongation is positive or negative. The significant refractive indices are: $\alpha = 1.615$ (shown crosswise), $\beta = 1.663$ (shown lengthwise and most commonly occurring index), $\gamma = >1.734$; all ± 0.002.

Microchemical Identification.—The microchemical method for the identification of sulfadiazine has already been described and will only be briefly referred to here (6). The method employs gold bromide in hydrochloric acid as the reagent, but instead of hydrobromic acid, a saturated solution of sodium bromide in water serves just as well. A drop of the sodium bromide solution is placed upon the object slide and a drop of 5% hydrochloric acid added. A few crystals of sulfadiazine are then added, followed by a drop of gold chloride solution, gently mixed into the preparation but not stirred vigorously. "Burrs" of very fine needles, with a brownish tinge, will gradually form on the surface of the test drop, increasing in size so that ultimately they can be readily discerned by the naked eye. Sodium sulfadiazine behaves similarly.

Sulfapyridine

Optical Crystallographic Data.—Sulfapyridine is a colorless crystalline powder without definite habit in so far as microscopic examination in ordinary light can determine. The significant refractive indices are: $\alpha = 1.680$, $\beta = 1.733$, $\gamma = >1.733$ (1). It is characteristic of the substance to show many fragments that match liquid, $\beta = 1.733$. All ± 0.003. According to Castle and Witt (3), this is the commonly occurring and stable form and the refractive indices they obtained very closely approximate these.

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Microchemical Identification.—In the microchemical identification of sulfapyridine, gold chloride is the test reagent (4). Its application results in the formation of yellow rods or blades, frequently occurring as x-shaped aggregates. Sodium sulfapyridine produces a similar precipitate, but the test-drop becomes brown at the periphery.

Sulfathiazole

Optical Crystallographic Data.—The habit of the grains is not significant. Like sulfapyridine, it also exhibits dimorphism (5). Hexagonal prisms form from *n*-propyl alcohol as the solvent. This is the stable form, having the following significant refractive indices: $\alpha = 1.674$, $\beta = 1.685$, $\gamma = >1.733$; all ± 0.002. The α and β values are most frequently observed. In convergent polarized light (crossed nicols), biaxial interference figures are commonly shown, especially perpendicular to the acute bisectrix, with positive optical character and a small axial angle. For determinative purposes, therefore, the frequent occurrence of the β-value, also interference figures showing both of the optic axes in the microscopic field, are the diagnostic optical feature for the substance.

Microchemical Identification.—Picric acid already has been described as a microchemical reagent for sulfathiazole (7). A more significant test is that with picrolonic acid. Approximately 2 mg. of sulfathiazole are mounted on an object slide and in a drop of 50% alcohol. After stirring the test-drop, an equal amount of dry picrolonic acid is added and also stirred in. A copious crystalline precipitate forms, consisting of fine needles arranged in circular aggregates. These can be allowed to dry on the object slide at room temperature for ultimate examination with the polarizing microscope.

Optical Crystallographic Properties of Crystalline Complex Formed with Picrolonic Acid.—With crossed nicols the needles show inclined extinction and negative elongation (with selenite plate). The needles invariably extinguish sharply, showing the following significant refractive indices: $\alpha = 1.582$ and $\gamma = >1.733$; both ± 0.002. The needles are too small to show interference figures.

Sulfaguanidine

Optical Crystallographic Data.—The powdered crystalline material does not show a significant habit in ordinary light, the fragments being irregular in shape. Many of these extinguish sharply with crossed nicols and others remain bright under the same conditions. The latter, in convergent polarized light (crossed nicols), show one optic axis in the biaxial interference figure. The significant refractive indices are: $\alpha = 1.606$, $\beta = 1.663$, $\gamma = 1.734$; all ± 0.002.

Microchemical Identification.—Sulfaguanidine failed to give any distinctive reaction products with any of the common microchemical reagents. However, it did form a crystalline complex with nitric acid, this being suitable for ultimate examination with the polarizing microscope. The crystalline precipitate is most conveniently prepared by dissolving about 5 mg. of the powdered sulfaguanidine in a drop of 10% nitric acid. The preparation is carefully stirred, then a drop of concentrated nitric acid is added and the preparation vigorously stirred to induce crystallization. Masses of elongated prismatic rods almost immediately form. The excess liquid may be removed with small strips of filter paper and the crystalline precipitate left to dry on the object slide at room temperature.

Optical Crystallographic Properties of Crystal-Complex Formed with Nitric Acid.—With polarized light, the extinction on the rods is usually parallel, occasionally some of them showing inclined extinction. The sign of elongation is positive. The significant refractive indices are: $\alpha = 1.578$ (commonly shown crosswise) and $\gamma = 1.663$ (commonly shown lengthwise); both ± 0.002.

Sulfamerizine

Optical Crystallographic Data.—Sulfamerizine as it usually occurs in tablets consists of very small fragments, too small to permit of interference figures that would aid in orientation. However, these crystalline fragments show strong double refraction with the following refractive indices: $\alpha = 1.587$ and $\gamma = 1.675$, both occurring frequently. Both ± 0.002.

Microchemical Identification.—One very characteristic microchemical test is that obtained with gold chloride and sodium bromide. A drop of 5% hydrochloric acid is placed on an object slide and a few fragments of sodium bromide added and stirred in. A small amount of sulfamerizine powder is then added and also stirred into the preparation. Finally, a drop of gold chloride reagent is added, whereupon numerous brown dendritic crystalline structures are observed.

Picrolonic acid also forms a crystalline complex with sulfamerizine, this consisting of numerous circular aggregates of small rods when a very small amount of picrolonic acid is mounted in a drop of 50% alcohol containing a very small amount of the test substance. These rods dry at room temperature on an object slide and can be subsequently examined with the polarizing microscope.

Optical Crystallographic Properties of Crystalline Complex Formed with Picrolonic Acid.—The rods show inclined extinction with crossed nicols and the sign of elongation is negative. Interference figures are not observed in convergent polarized light (crossed nicols). The significant refractive indices are: $\alpha = 1.572$, $n_t = 1.733$, $\gamma = >1.733$; both ± 0.002.

SUMMARY

The optical crystallographic properties of sulfanilamide, sulfadiazine, sulfapyridine, sulfathiazole, sulfaguanidine, and sulfamerizine have been described. Microchemical tests for these sulfonamides are also given, together with optical crystallographic descriptions of the crystalline reaction products resulting from these tests.

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A Contribution to the Chemistry of Sulfamide*

By C. A. ANDRESON† and C. E. MILLER‡

The preparation of sulfamide by the reaction between ammonia and sulfuryl chloride appears to be the only practical method. A simplified procedure using this method is described.

CONSIDERABLE interest has been shown in the sulfur dioxide analog of urea, which is known in commercial circles as sulfamide. Because of the importance of urea to the medical profession, many investigators have sought to develop a practical method of preparing sulfamide economically in order that it might be thoroughly evaluated. A survey of the literature (1–5) shows that most investigators placed emphasis on the reaction between sulfuryl chloride and ammonia. In recent years Degering and Gross (6) have proposed a reaction between liquid ammonia and sulfuryl chloride at temperatures below −40°. The autoclave described and low-temperature facilities needed by Degering and Gross are not always available in the average laboratory. The purpose of this paper was to investigate reactions heretofore untried and to develop a simplified method of preparation which does not require below zero temperatures.

EXPERIMENTAL

Organic Reactions

Several organic reactions were tried using sulfuryl chloride as the starting material. In the first reaction a mole-for-mole ratio of sulfuryl chloride and potassium phthalimide using petroleum ether as a diluent were mixed and after the initial reaction was completed the solid material was worked up in the usual manner. Positive qualitative tests for sulfur, halogen, and nitrogen were obtained, indicating that the reaction had proceeded according to the equation in the adjacent column.

Attempts to subject the phthalimidochlorosulfone to ammonolysis with liquid ammonia, gaseous ammonia, hydrazine hydrate, and liquid hydrazine were unsuccessful.

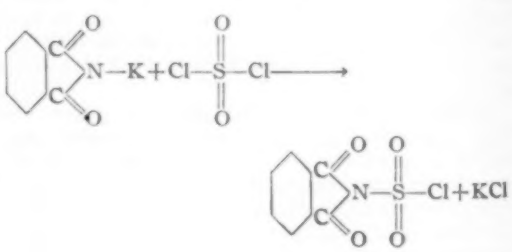
A second reaction was carried out in which potassium succinimide was substituted in place of the phthalimide salt. However, after several hours of refluxing, sulfuryl chloride was recovered unchanged. It was concluded that potassium succinimide did not react with sulfuryl chloride.

Inorganic Reactions

The availability of ammonium sulfamate and its cheapness made it seem worth while to study this compound with the hope of effecting its dehydration to yield the desired product, sulfamide. Ammonium sulfamate was heated under reflux with glacial acetic acid, acetic anhydride, toluene, chlorobenzene, nitromethane, and nitroethane but in no case was dehydration effected. Ammonium sulfamate was also heated without a diluent at 100° at a vacuum of 1 mm. for ten hours but with no change in the ammonium salt. It was concluded that ammonium sulfamate did not dehydrate under the described conditions.

The possibility of amidation of sulfamic acid was next considered. A series of reactions was tried between sulfamic acid and acetamide, phthalimide, and sodium amide, using catalysts such as iodine, phosphoric acid, sodium hydroxide, and benzoyl peroxide. In no case was the desired product isolated.

The reaction between sulfur trioxide and liquid hydrazine was carried out both at room temperature and at 0°. Mole ratio quantities of sulfur trioxide, generated from 25% oleum and liquid hydrazine, prepared from hydrazine hydrate reacted very vigorously at both temperatures and ammonium sulfate was the final product isolated. No further study of this reaction was made.



Consideration was next given to the addition of gaseous ammonia to sulfuryl chloride using diluents such as halogenated hydrocarbons, nitroparaffins, and hydrocarbons. The temperature was main-

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tained low enough by the use of an ice bath to insure maximum utilization of the ammonia. The results obtained in all runs utilizing halogenated hydrocarbons and nitroparaffins were negative, even in the presence of ultraviolet light. However, when using petroleum ether as the diluent and with the temperature maintained at from 0–10°, sulfamide in yields of from 18% to 25%, based on the sulfuryl chloride used, was obtained.

The possibility of impurities in the sulfuryl chloride having a detrimental effect on the formation of sulfamide was considered, in view of the reddish brown color present in the crude sulfamide obtained. The commercial grade of sulfuryl chloride used in this study contained about 5% of thionyl chloride. Accordingly amounts of thionyl chloride were added to the sulfuryl chloride prior to use to give concentrations ranging from 10% to 50%. It was ascertained that the presence of 10% of thionyl chloride as an impurity in sulfuryl chloride would entirely prevent the isolation of sulfamide, using petroleum ether as a diluent. The higher the percentage of thionyl chloride, the more viscous the resulting end product, and in the higher ranges of thionyl chloride impurities, this syrupy viscous liquid persisted for months.

The results of this part of the investigation between gaseous ammonia and sulfuryl chloride proved that sulfamide could be prepared in fair yields, using petroleum ether as the diluent. Our emphasis was placed on the developing of a suitable laboratory procedure, in which the temperature need not be lower than that obtained by ice and water. The method developed by the authors is presented in detail:

The necessary starting materials, anhydrous ammonia, sulfuryl chloride, and petroleum ether were laboratory stock and were used without further purification. In a 500-cc. three-necked flask fitted with a thermometer, mechanical stirrer, and gas inlet tube was placed 10 Gm. of sulfuryl chloride and 200 cc. of petroleum ether. The flask was surrounded with an ice-water bath, the stirrer started, and stirring continued until the contents of the flask had cooled to 0°. Anhydrous ammonia was then added above the surface of the stirred liquid at a rate consistent to keep the temperature from exceeding 10°. It was found that stirring at about 1500 r. p. m. gave maximum contact between the reactants. The anhydrous ammonia was added continuously until the temperature dropped to 0°. About forty-five minutes was required for this temperature drop to occur and this was taken as an indication of the end of the reaction.

The contents of the flask were allowed to warm to room temperature, the solid material removed by filtration, and dried by suction. The resulting powder was dissolved in a minimum of distilled water, slightly acidified with hydrochloric acid, and allowed to stand at room temperature for three days. The solvent was removed by desiccation over sulfuric acid, and the crude sulfamide mixed with dry clean sand and extracted with acetone for three hours. Removal of the acetone yielded crystalline sulfamide in amounts approaching 70%. Analysis of the material obtained by the method described is given herewith:

% N sought	29.15
% N found	28.86
m. p. corrected	92.1°

SUMMARY

1. Attempts to prepare sulfamide by any reaction other than that between ammonia and sulfuryl chloride were unsuccessful. However, proper control of temperature, acid hydrolysis, and the use of petroleum ether as a diluent, produced sulfamide in yields of about 70 per cent without employing extremely low temperatures or elaborate equipment.

2. The presence of 10 per cent or more of thionyl chloride as an impurity in sulfuryl chloride is detrimental to the formation of sulfamide.

3. The use of sand as a diluent to prevent packing in the final acetone extraction was found to be advantageous.

4. Nitrogen analysis of the compound, sulfamide, is reported for the first time.

5. Acknowledgment is given to Hooker Electrochemical Company for generously supplying the sulfuryl and thionyl chlorides used.

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The Chromatographic Isolation of the Trihydroxymethylanthraquinones of Cascara Sagrada*, †

By MELVIN R. GIBSON and ARTHUR E. SCHWARTING‡

The chromatographic isolation of the isomeric trihydroxymethylanthraquinones of Cascara Sagrada was investigated. It was found that under a given set of conditions the three isomers would separate into three distinct bands on the chromatographic tube. This isolation, in conjunction with spectrophotometry, was used as the basis for a method of quantitative analysis of Cascara Sagrada preparations.

SINCE their introduction to the field of medicinals, the preparations of Cascara Sagrada have undergone varied and extensive investigation to determine their active principles and to find an accurate method of quantitative analysis. The problem of finding one or a group of specific constituents that are wholly responsible for the action of this drug is unsolved. A recent conclusion (1) is that the physiological action is a result of several principles acting synergistically and that the trihydroxymethylanthraquinones play a role in this mechanism.

A major problem in the analysis of Cascara Sagrada and its preparations is the isolation of the anthraquinones. The use of adsorption as a method of analysis (2) presented the possibility that the anthraquinones themselves might be adsorbed, eluted, and determined, using methods of chromatography.

PREPARATION OF MATERIALS

For comparative determinations in a chromatographic isolation it was first necessary to obtain the respective anthraquinones in pure form.

Emodin (1,6,8-trihydroxy-3-methylanthraquinone) was prepared by the Jowett (3) process and by a simple method evolved in the present work. This latter method follows. One hundred sixty grams of the powdered bark was refluxed with 6400 cc. of chloroform. On evaporation of this solution to 500 cc., nearly pure emodin was obtained. Recrystallization and sublimation of the material under reduced pressure produced emodin in pure form.

Aloe-emodin (3-(hydroxymethyl)-1,8-dihydroxyanthraquinone) was prepared by the process of Liddell, King, and Beal (1).

Iso-emodin (3,5,8-trihydroxy-2-methylanthraquinone) was prepared by a modification of the process of Green, King, and Beal (4). Using the materials and procedure of this process it was impossible to obtain iso-emodin. The modification of the process by which iso-emodin was obtained follows: The glacial acetic acid solution was diluted with 100 cc. of water to cause precipitation. This preparation was extracted with successive portions of chloroform until Borntraeger tests (5) appeared negative. A total volume of 1125 cc. was required. This solution was passed through a chromatographic column packed with 1 part of Micron Brand Magnesia and three parts of Celite. The anthraquinone of the lower red layer formed on the column, when shaken out with chloroform after solution of the magnesia with 10% hydrochloric acid, did not give a red coloration with ammonium hydroxide. Since iso-emodin is the only anthraquinone in Cascara Sagrada which reacts in this manner, the adsorbed material was considered to be iso-emodin and was further purified in the following manner. The chloroform solution obtained by elution was evaporated to dryness and carbon disulfide was added to the residue to remove a waxy material preventing crystallization. The residue was then dissolved in 10% potassium hydroxide solution, the solution acidified and re-extracted with chloroform. The residue obtained by evaporating this solution was sublimed under reduced pressure. A bright orange sublimate resulted. The crystals were washed with carbon disulfide until microscopic examination revealed a "dry" crystalline product. This iso-emodin obtained had a melting point of 179–180°. This constant is not in agreement with that reported for iso-emodin by Green, King, and Beal (4).

Spectrophotometric Examination.—Each of the anthraquinones obtained was examined spectrophotometrically in a similar manner. Solutions of each in alcohol were prepared with the following concentrations: 0.01 mg. per cc., 0.008 mg. per cc., 0.005 mg. per cc., and 0.0025 mg. per cc. These solutions were examined in a Coleman Universal Spectrophotometer using the null method at 10 mμ

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intervals between the wave lengths 355–650 mμ. Alcohol was used as the blank and the samples were contained in square cuvettes 1.3 cm. in diameter. The spectrophotometric curve of the 0.008 mg. per cc. dilution of each anthraquinone is graphed in Fig. 1. It was also found that all three anthraquinones conform to Beer's Law (i.e., the absorptance by a solution is directly proportional to the concentration of the solute).

Figure 1 illustrates the points of maximum absorption of iso-emodin and aloe-emodin at 430 mμ, and emodin at 440 mμ. These characteristics were found to be consistent through all four dilutions.

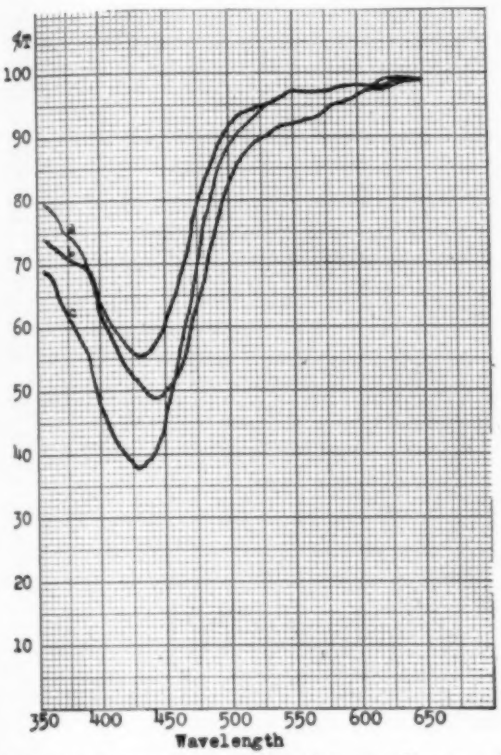

Fig. 1—Comparative Transmittance Curves of the Anthraquinones.
a, iso-emodin; *b*, emodin; *c*, aloe-emodin.

CHROMATOGRAPHY

With adequate quantities of the anthraquinones at hand and having a knowledge of their spectrophotometric properties it was possible to interpret properly the effectiveness of chromatographic isolation.

Chromatograms of extracts of Cascara Sagrada were first prepared by Ernst and Weiner (6) using alcoholic extracts of the bark passed through alumina. The alumina had been previously washed with water and the chromatograms were developed with alcohol Ether was used as the eluant. A partial elution was obtained.

The investigation of the chromatography of the anthraquinones of Cascara Sagrada revolved around finding a suitable liquid from which differential adsorption would take place, an effective adsorbing media, and an efficient eluant. Since the field of chromatography is still largely in a state of trial and error, only general rules could be followed. The recorded work involves a series of trials based upon a meager store of general procedures and is largely dependent upon a systematic elimination or amplification of the many possibilities for obtaining an effective use of the chromatogram as an isolating medium.

EXPERIMENTAL

Alumina was first chosen as an adsorbing agent following the suggestion of Strain (7) for compounds of this general class. Of a number of organic solvents used, alcoholic extracts of the bark of Cascara Sagrada demonstrated a crude differentiation of the red layer, originally formed, into indistinct bands of various shades of red. Acidification of the alcoholic extract gave a sharper degree of separation. Development of the chromatogram with a series of solvents was successful only with alcoholic solutions with a slightly greater acidity. Red, orange, and pink layers, poorly differentiated, were observed under these conditions. Elution of the dry powder was successful only with a 10% solution of potassium hydroxide.

Bentonite, kaolin, and a brand of adsorption magnesia, and the latter in combination with various proportions of Celite were tested as absorbing agents. The magnesia-celite combination was highly successful. The bentonite, kaolin, and the magnesia offered no advantages. Using a 3:1 mixture of Celite (Johns-Manville Celite Number 501) and magnesia (Micron Brand Adsorptive Magnesia Number 2641, Westvaco Chlorine Products Company), respectively, and a dilute chloroform extract of a U. S. P. Extract of Cascara Sagrada a chromatogram was obtained as follows: Five grams of the Extract was refluxed for one hour with 250 cc. of chloroform. The solution obtained was passed through a 38-mm. chromatographic tube packed with the magnesia-celite mixture. A red band 2 mm. wide resulted. The amount of free anthraquinones present was too small to afford accurate judgment of the process. To obtain a larger quantity of anthraquinones, as aglycones, the marc was refluxed for two and one-half hours with 250 cc. of chloroform and 50 cc. of a 25% sulfuric acid solution. The chloroform solution was passed through a magnesia-celite column 38 mm. in diameter and the chromatogram appeared as follows: 4 mm. red-brown, 4 mm. deep red, 4 mm. red-orange, 2 mm. red-tan and 2 mm. pink. The layers were distinctly differentiated.

In order to substantiate differential adsorption of anthraquinones a dilute chloroform solution of the pure anthraquinones was prepared and passed through a chromatographic tube containing the

magnesia-celite mixture. Upon continued washing with chloroform over a period of a week a differentiation of the initial red layer was evident. A deep red layer was followed successively by an orange and a pink layer. The red-tan layer occurring in the extract chromatogram was wanting. By preparing chromatograms of emodin and iso-emodin and of emodin and aloe-emodin and comparing the colors, the chromatograms prepared from the extract suggested the red layer as representing emodin, the orange, aloe-emodin, and the pink, iso-emodin. A chromatogram prepared from the extract is shown in Fig. 2.

Elution of the several layers was accomplished by treating the dry powder with 10% hydrochloric acid to effect solution of the magnesia. The anthraquinones were then shaken out with chloroform.

Fig. 2.—A Chromatogram Showing Differentiation of the Anthraquinones.

For proof of the separation, the anthraquinones were obtained in crystalline form from these layers.

Isolation

Free Anthraquinone Separation.—A 5.25-L. chloroform extract obtained from 105 Gm. of a U. S. P. Extract of Cascara Sagrada was used. The resulting chromatogram appeared as follows: 7 mm. red-brown, 15 mm. deep red, 10 mm. orange, 2 mm. pink. With the large quantity of liquid used, deactivation of the magnesia was evident. Each of the layers was separated, eluted and each solution passed through chromatographic tubes 12 mm. in diameter. This process of purification proved that all layers were homogeneous except the top red-brown layer. In successive chromatograms prepared from this layer it showed differentiation into three layers comparable to the parent chromatogram. The red-brown layer was proved to be a mixture of all three anthraquinones, the formation of which precedes the differentiation of the individual anthraquinones. This is common in many chromatograms of plant principles (8).

The red, the orange, and the pink layers of the second chromatograms were separated, eluted, and examined spectrophotometrically. The results of spectrophotometric examination of alcoholic solutions of products of these layers are shown in Fig. 3.

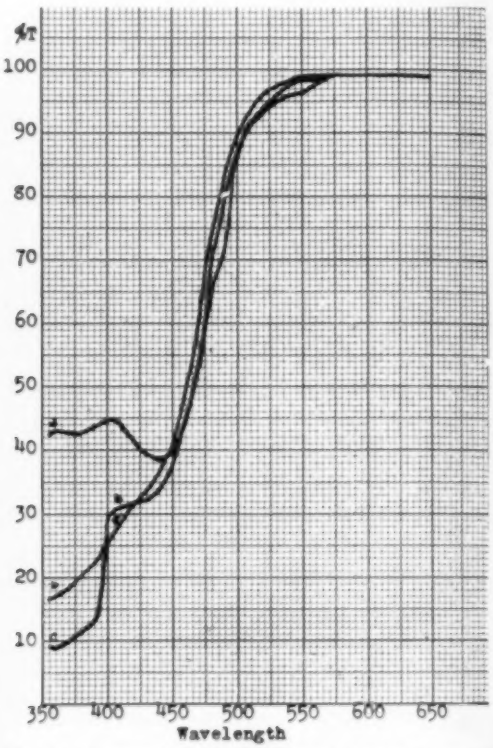

Fig. 3.—Transmittance Curves of Eluted Layers of Free Anthraquinones.
a, red layer; *b*, pink layer; *c*, orange layer.

These results illustrate that chromatographic differentiation occurred but that a contaminant was affecting the results of the lower regions of the spectrum.

Combined Anthraquinone Separation.—One liter of a chloroform extract of a U. S. P. Extract of Cascara Sagrada obtained by hydrolysis, in the presence of chloroform, of the marc from the above extraction was used in preparing a chromatogram. The chromatogram appeared as follows: 20 mm. red-brown, 40 mm. deep red, 5 mm. orange, and 7 mm. pink. The layers were eluted and chloroform solutions were passed through 12-mm. chromatographic tubes for further purification. The homogeneous layers of these second chromatograms of each layer

were eluted and the residue sublimed under reduced pressure. The sublimates were washed with carbon disulfide and the residues were re-sublimed under reduced pressure. The sublimate in each case was crystalline. Each was dissolved in alcohol in a known concentration and examined spectrophotometrically. The results are shown in Fig. 4. The similarity of the transmittance curves with those of the pure anthraquinones indicates the separation of the respective anthraquinones. Melting point determinations of each of the products were in agreement with the pure products.

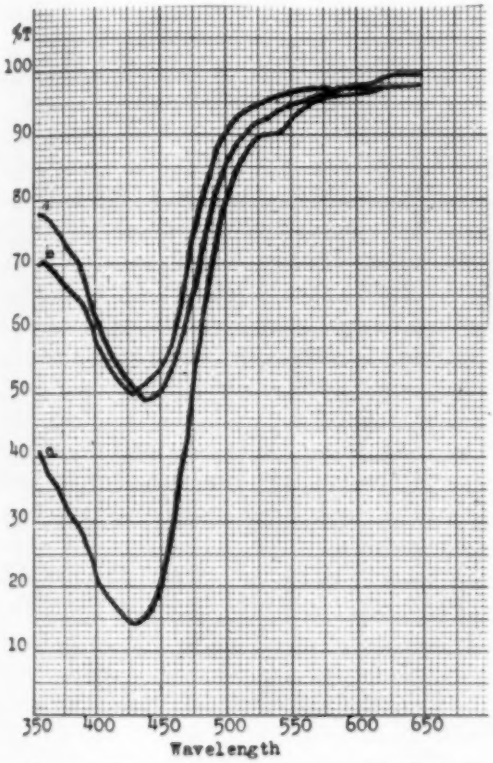

Fig. 4.—Transmittance Curves of Eluted and Sublimed Layers of Aglycones of Combined Anthraquinones.
a, red layer (emodin); *b*, pink layer (iso-emodin); *c*, orange layer (aloe-emodin).

CALCULATIONS OF RESULTS

It was found that by continually repeating chromatograms the anthraquinones could be completely purified, and that the sublimation process expedites obtaining each in pure form. For quantitative work neither of the procedures is practical. Therefore, if chromatography is used as a means of quantitative isolation of the anthraquinones it is important to know the nature of any impurity appearing in first-run chromatograms. A pale yellow layer appeared directly below the adsorbed anthraquinones on all chromatographic columns. This substance was characterized by Ernst and Weiner (6) as an anthranol.

In the development of the chromatograms it was demonstrated that more of this yellow material could be washed from the adsorbed anthraquinone layer on successive chromatograms. The spectral transmittance curve of this yellow layer showed maximum absorption in the 350–400 mμ area. A quantity of this yellow layer was eluted and the residue of the eluate appeared as a red-brown powder, crystalline in nature. Solutions of three concentrations were prepared and, on spectrophotometric examination, were found to conform to Beer's Law using the transmittance values at 440 mμ. Therefore, although the compound was not characterized, it may be treated as a chemical entity.

To determine a method of calculating results when the yellow material is a contaminant, solutions of known quantities of mixtures of the three anthraquinones were first prepared and examined spectrophotometrically. As previously shown, the anthraquinones demonstrated only slightly different spectral transmittance curves. This follows the conclusion reached by Elvidge (9) that compounds of a homologous series usually show similar spectral transmittance curves differing only slightly in areas of maximum absorption. The results are shown in Table I.

TABLE I.—TRANSMITTANCE OF MIXTURES OF ANTHRAQUINONES OF KNOWN CONCENTRATION

Wave Length, mμ	Emodin 40%, Aloe-Emodin 60%	Emodin 80%, Iso-Emodin 20%	Emodin 33⅓%, Aloe-Emodin 33⅓%, Iso-Emodin 33⅓%
355	68.5	73.5	68.0
360	68.0	72.0	67.5
370	65.0	69.5	64.0
380	61.5	67.7	61.0
390	58.5	65.0	58.5
400	50.0	57.0	50.5
410	45.5	52.5	46.0
420	41.3	48.0	42.5
430	39.0	45.0	40.0
440	38.5	43.5	40.0
450	40.5	44.5	42.7
460	46.0	49.0	49.0
470	56.0	56.0	58.0
480	66.0	65.0	67.3
490	76.5	74.3	77.0
500	83.0	81.0	83.5
510	88.0	85.5	86.5
520	90.5	88.0	89.0
530	93.5	90.0	91.0
540	94.5	91.0	93.0
550	95.0	92.0	94.0
560	96.0	92.5	94.0
570	97.0	94.0	95.0
580	97.0	94.5	95.5
590	97.0	96.0	96.5
600	99.0	97.0	96.5
610	99.0	97.5	98.0
620	99.0	98.0	98.0
630	99.0	98.5	98.0
640	99.0	98.5	98.0
650	99.0	98.5	98.0

The results of calculating the total solute content on the emodin extinction coefficient basis are shown in Table II.

TABLE II.—RESULTS OF CALCULATING TOTAL ANTHRAQUINONE CONTENT ON EMODIN EXTINCTION COEFFICIENT BASIS

Composition of Mixtures	Total Volume, Cc.	Total Amount of Solute, Mg.	Amount of Solute Calculated from Transmittance, Mg.	Error, Mg.
40% Aloe-emodin, 60% Emodin	10	0.10	0.1070	0.007
80% Emodin, 20% Iso-emodin	10	0.10	0.0933	0.0067
33⅓% Emodin, 33⅓ Aloe-emodin, 33⅓% Iso-emodin	6	0.06	0.0616	0.0016

The formula $C = \frac{\log_{10} Io/I}{Kb}$ was used in calculating the emodin extinction coefficient (K) at 440 mμ based on the transmittance of emodin (49.0%) for a solution of known concentration (0.008 mg. per cc.). In the formula C is equal to the concentration, expressed in mg. per cc.; $\log_{10} Io/I$ is the logarithm of the ratio of incident to emergent light; K is the extinction coefficient expressed in mg. per cc.; and b is the thickness of the absorption cell in cm. The specific extinction coefficient for emodin is 29.784.

The results indicate that total anthraquinones may be calculated on the basis of the extinction coefficient of emodin.

To determine to what extent the yellow material affected the transmittance readings of anthraquinones at 440 mμ a solution of known quantities of all three anthraquinones and the yellow material was prepared and examined spectrophotometrically as shown in the second column of Table III.

The facts illustrated in the tables indicate that the total anthraquinones of a solution may be calculated on the basis of a single extinction coefficient and that the yellow material, showing slight absorption at 440 mμ might contribute part of the optical density of a solution when it is combined with the anthraquinones. Total quantities of anthraquinones and yellow material were, therefore, calculated simultaneously, using the binary system of spectrocomputation (10). The specific extinction coefficients of emodin were based on a 0.01 mg. per cc. dilution showing 70.5% transmittance at 370 mμ and 42.5% transmittance at 440 mμ. A solution of the yellow material at the same dilution gave 85.0% transmittance at 370 mμ and 92.5% transmittance at 440 mμ. The extinction coefficients for emodin at 370 and 440 mμ were 11.667 and 28.572, respectively, and for the yellow material 5.4161 and 2.6023.

The quantities of each of the constituents in the solution and the results calculated on the basis of the extinction coefficient for emodin and on the binary system are shown in Table IV. The spectral transmittance data of two dilutions are shown in Table III.

The results of the analysis of the first solution illustrate that the difference between the two methods of computation presents little error. The second solution, an extreme case, illustrates a similar result. The results obtained by calculating on the binary system are not significantly different from the results obtained by calculating on the basis of the emodin extinction coefficient alone. The error in the latter case is within the experimental error and does not justify use of the more complicated binary system.

TABLE III.—TRANSMITTANCE OF MIXTURES OF ANTHRAQUINONES AND YELLOW MATERIAL OF KNOWN CONCENTRATION

Wave Length, mμ	Equal Quantities of Anthraquinones and Yellow Material	80% Yellow Material, 20% Emodin
355	70.0	70.5
360	69.0	70.5
370	66.5	70.5
380	63.5	71.5
390	61.5	71.5
400	55.0	71.5
410	50.5	69.5
420	47.0	68.0
430	46.0	67.0
440	46.0	67.0
450	48.3	68.0
460	54.5	71.0
470	62.5	75.5
480	72.0	81.0
490	81.5	86.0
500	87.0	90.0
510	91.5	92.0
520	93.0	93.0
530	94.0	94.5
540	96.5	94.5
550	97.0	95.0
560	98.0	95.7
570	98.0	96.0
580	98.5	97.0
590	98.5	97.0
600	98.5	97.5
610	99.0	97.5
620	99.0	97.5
630	99.0	98.0
640	99.0	98.0
650	99.0	98.5

SUMMARY AND CONCLUSIONS

The results of these experiments demonstrate that while chromatography does not in the preparation of a single column, effect complete isolation of the anthraquinones of Cascara Sagrada, the impurity obtained on elution of the red layers of the chromatogram does not appreciably affect the absorption of the anthraquinones in the area utilized for measuring their spectral trans-

TABLE IV—CONCENTRATION OF MIXTURES OF ANTHRAQUINONES AND YELLOW MATERIAL CALCULATED ON EMODIN BASIS AND BY BINARY SYSTEM

Composition of Mixtures	Total Volume, Cc.	Total Amount of Solute, Mg.	Anthraquinones Calculated on Emodin Basis, Mg.	Error on Emodin Basis, Mg.	Anthraquinones by Binary System, Mg.	Error in Binary System, Mg.
25% yellow material 25% iso-emodin 25% aloe-emodin 25% emodin	7	0.08	0.0612	0.0012	0.0588	0.0012
80% yellow material 20% emodin	6	0.10	0.0266	0.0066	0.0198	0.0002

mittance. The use of chromatography as a method of isolation of the anthraquinones and spectrophotometry as a method of quantitative analysis, therefore, is feasible.

The presence of the three anthraquinones was substantiated. However, at no time was a layer of material obtained which could be interpreted to be chrysophanic acid. Chrysophanic acid was reported to be present in Cascara Sagrada by Le Prince (11) and Liddell, King, and Beal (1). Jowett (3) following the procedure of Le Prince did not obtain chrysophanic acid and in other attempts failed to procure it. The authors repeated the work of Liddell, King, and Beal and were unsuccessful in obtaining chrysophanic acid. A review of the literature concerning chrysophanic acid revealed interesting data. It is known that chrysarobin is converted readily to chrysophanic acid by the air, particularly under alkaline condition. Also, the melting points reported for chrysarobin and iso-emodin have interesting parallels. The "Handbook of Chemistry and Physics" (12) lists 170–178° and 205–210° as melting points for chrysarobin. Karrer (13) lists a melting point of 204° for this compound. Jowett (3) reported iso-emodin as melting at 180°. Green, King, and Beal (4) report iso-emodin as having a melting point of 215° and Keimatsu and Hirano (14) established this value by synthesis. One might well interpret these figures as indicating that chrysarobin and iso-emodin are being confused. The compound melting at near 180° was considered to be iso-emodin. Crystallographic and polarographic measurements will resolve these data. It is apparent that chrysophanic acid is not preexistent in the bark of Cascara Sagrada but it is probable that it is formed during extraction or drying procedures.

In this study the great utility of chromatography to the field of chemical investigation was illustrated. The amount of tedious preliminary investigation necessary illustrates a need for pure chemical and physical research in this field to establish fundamental rules for its intelligent use. The results of successful chromatography are spectacular. Its use as a method of chemical isolation will only be generally accepted when the knowledge of the fundamentals of its mechanism are known and can be applied to obtain such results.

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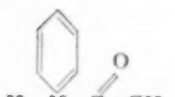
A Contribution to the Pharmacodynamics and Chemistry of Glycinanilide*

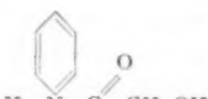
By HARRY K. IWAMOTO, ELAINE B. GABY,† RUTH D. MUSSER, and C. JELLEFF CARR

This first approximation of the pharmacodynamics of glycinanilide indicates that the compound is less desirable as a therapeutic analgesic than is acetanilide.

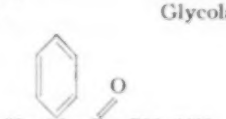
IN OUR investigations of compounds evoking analgesic activity, glycolanilide was prepared and studied (1). This compound was found to be a rapid producer of methemoglobin in man and hence not available for use as an analgesic. Our studies were next directed to glycinanilide. The relationship of these compounds to acetanilide may be observed from the following formulas:



Acetanilide



Glycolanilide



Glycinanilide

Although the acetophenetidin analog of glycinanilide has been studied on animals and man under the name of Phenokoll (2), we were unable to find any systematic study of the pharmacodynamics of glycinanilide.

EXPERIMENTAL

Synthesis.—The product used in these studies was prepared by a method similar in principle to one (3) known in the literature (4–8). By the application of catalytic hydrogenation procedure, extensively studied by Hartung and co-workers for the preparation of primary amino compounds from the corresponding oximinoketones (9) to oximinoacetanilide by an electrolytic method as described by Karrer and Haebler (3), it was possible to obtain glycinanilide hydrochloride. The reaction is straightforward with a minimum of involvements, and the yields are good. They increase with manipulative refinements as the reduction proceeds almost quantitatively.

In a typical experiment, 16.4 Gm. (0.1 mole) of pure isonitrosoacetanilide (10) was dissolved in 150 cc. of ethanol containing 3 equivalents of HCl (or in a solvent mixture of 150 cc. of absolute ethanol and 30 cc. of concentrated hydrochloric acid), and reduced with 5 Gm. of palladinized carbon catalyst. On a Parr apparatus, complete hydrogenation was usually accomplished in thirty minutes, depending upon the activity of the catalyst. The filtrate was freed from the solvent in a desiccator over anhydrous calcium chloride and sodium hydroxide until a dry residue was obtained. The product, orange-yellow in color, melted slowly over a temperature range of 175–195°, and weighed approximately 18.7 Gm., representing nearly a quantitative conversion.

The crude glycinanilide hydrochloride was purified by decolorizing with "Nuchar" in anhydrous ethyl acetate or absolute ethanol, followed by the addition of sufficient acetone until incipient signs of crystallization became perceptible, and finally completing the crystallization by refrigeration. White plates or leaflets were obtained in 14.4 Gm. yield (77%), having a melting point range of 197–203° (uncor.); literature: 190–195° (4). A mixed melting point of our product with a sample of glycinanilide hydrochloride prepared according to Hill and Kelsey (3) showed no depression. A picrate of our product melted with decomposition at 186°; literature: 186° (8).

Oral Toxicity (Rats).—Glycinanilide hydrochloride was administered to white rats by stomach tube, using a 10 per cent solution. The animals were fasted previously for twenty-four hours. The deaths occurring in twelve hours were recorded. With 15 animals, doses up to 1000 mg./Kg. produced only transient cyanosis but no other apparent symptoms over a forty-eight-hour period of observation.

Blood Pressure Studies (Dogs).—The effect of glycinanilide upon the blood pressure of the dog was studied. The animals were anesthetized with ether and the compound injected intravenously. Eight animals were employed. Volumes of 2.5 to 5 cc. of a 5 per cent solution of the hydrochloride of glycinanilide produced a prompt fall in blood pressure of short duration; this was followed by a transient rise in pressure. The magnitude of the effect appeared to be a function of dose and some degree of tolerance was elicited upon repetitious injections. During the period of the blood pressure response the respiration was depressed in rate and amplitude.

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Electrocardiographic Studies (Dogs).—On 5 of the dogs used for blood pressure studies, electrocardiograms were taken under ether anesthesia, prior to and immediately following the injection of the glycinanilide. There were no significant changes produced in the rate, rhythm, or form of the electrocardiogram in Leads I, II, or III.

Chronic Toxicity (Rats).—Forty young male white rats were divided into 4 groups of 10 each. One group served as a control and was fed a normal laboratory ration. Two groups received in their food 0.2% and 0.5%, respectively, of glycinanilide. The fourth group received 0.5% acetanilide in their food. The animals in all groups grew according to the normal growth pattern. The rats receiving the glycinanilide developed cyanosis (0.5% 10 days, 0.2% 15 days). The spectrophotometric examination of blood taken from cardiac puncture showed no spectral band for methemoglobin, but bands in the reduced hemoglobin region were observed.

The experiment was continued for one month. At the end of this period 4 animals in the glycinanilide group and one control animal were sacrificed for histologic study of their viscera. Their kidneys, liver, and spleen showed no significant variation from the normal.

Analgesic Studies.—The analgesic properties of glycinanilide were determined in the rat by the Thorp (11) method, using the Wolff–Hardy–Goodell apparatus. In 15 animals the pain threshold was increased 7% one-half to one hour after 500 mg. per Kg. of glycinanilide were administered by stomach tube. Acetanilide in the same dosage elevated the pain threshold 52% to 10 animals. Morphine sulfate in a dose of 3 mg. per Kg. subcutaneously produced an elevation of the pain threshold of 22 per cent in these same animals.

DISCUSSION

Glycinanilide hydrochloride is a soluble compound of the acetanilide type. The catalytic method affords a facile synthesis with fair yields. However, it is necessary to purify the product with care; since incomplete hydrogenation is attended by intramolecular condensation products (12) in trace amounts sufficient to mottle the product on standing, impart a dull green turbidity to its freshly prepared solution and acutely affect the toxicity data even though the melting point of the glycinanilide may not betray its presence. Its effect upon oxyhemoglobin in a rat appears to be more pronounced than that of acetanilide and comparable to the effect of glycolanilide. The analgesic effect of glycinanilide is inferior to that of acetanilide.

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Book Reviews

Essentials of Prescription Writing. By CARY EGGLESTON. Eighth edition. W. B. Saunders Company, Philadelphia, 1947. viii + 155 pp. 9 x 17 cm. Price, $2.

When a guidebook in so prosaic a field, for such it is to the average physician, has attained its eighth writing, there is little which a reviewer can say other than "well done." The 8th edition of this useful little book follows the same pattern as former editions. New examples are often used to illustrate a point and the revision has been changed to be in keeping with the latest revisions of the official compendia. Unfortunately, on page 3 the author gives the impression that N. F. VII is now official and he states that its revision has been at irregular intervals. The National Formulary has had the same projected publication date as the U. S. P., but, like the U. S. P., necessary delays have, at times, given the impression of irregularity.

Schimmel 1945 Annual Report on Essential Oils, Aromatic Chemicals, and Related Materials. SCHIMMEL and COMPANY, INC., 601 W. 26th Street, New York 1, N. Y., 1948. 127 pp. Illustrated. 15 x 23 cm. Price, $3.

In this report an apparently successful attempt has been made to present in practically the same style as in previous reports pertinent information on essential oils and related materials. Commercial and scientific developments are discussed and notes on scientific research in the field of aromatics and related products are presented. The usual statistical notes are included and the value of the report is enhanced by a bibliography and a satisfactory index.

It is expected that the 1946 report will be completed and published in the near future. All who are interested in essential oils will be glad to know that this valuable publication will be continued.

Bergey's Manual of Determinative Bacteriology. By ROBERT S. BREED, E. G. D. MURRAY, and A. PARKER HITCHENS. Sixth edition. The Williams & Wilkins Company, Baltimore, Maryland, 1948. xvi + 1529 pp. 15 x 23.5 cm. Price, $15.

This well-known manual for many years has been used as a standard reference in the field of bacteriology. The sixth edition has been carefully revised and many improvements over previous editions are quite obvious. The current edition has been completely re-set in a new double column format which permits more material on each page. Numerous changes have been made in the outline classification. Nearly 300 new species are described. In every instance new presentations were prepared by specialists, under the direction of the Editorial Board. A completely new feature is the Source and Habitat Index which should be of particular significance to all who are interested in determinative bacteriology. The Genus and Species Index has been brought up to date and includes synonyms and incompletely described species. The completion of this volume which has been more than four years in press represents a valuable contribution to the science of bacteriology.

Combat Scientists. By LINCOLN R. THIESMEYER and JOHN E. BURCHARD. Atlantic—Little, Brown and Company, Boston, 1947. xix + 412 pp. 14 x 21 cm. Price, $5.

That scientists played a major part in the World War II is known by all, but only now can be told in some measure how these amazing developments took place. Most of us think of scientists only in connection with laboratories. *Combat Scientists* tells of the activities of those scientists who actually participated in the war at levels of top echelons in the Pentagon and the foxholes—the Office of Field Services, OFS, of the OSRD.

From one end of the world to the other OFS scientists observed men and matériel under combat conditions. Riding jeeps, submarines, carriers, and the bellies of C-54's they participated in the operational analysis of all sorts of military functions. How to make fire power more effective, prevent the disease "jungle rot," spray DDT from Naval vessels, stop the kamikazes, determine the morale effect of our weapons on the enemy and the enemies' weapons on our men—all of these stories and many more can now be told.

Pharmacists will be especially interested in the project of Dr. Hopkins, eminent medical mycologist, in his studies of fungus diseases in the Pacific. It was his development of the use of undecylenic acid and propionate ointments which increased the comfort of the men who often waded through foul rice paddies and other conditions conducive to mycoses of the most insidious types.

The Essential Oils. By ERNEST GUENTHER. Vol. I. D. Van Nostrand Company, Inc., New York, 1948. xvi + 427 pp. 55 illustrations. 15 x 23.5 cm. Price, $6.

For some time the need for an up-to-date comprehensive treatise on essential oils has been recognized. This volume, the first of a series, together with the succeeding volumes already planned, should supply this need.

In the first volume the history, chemistry, biology, origin, and functions of essential oils, methods of production and analysis are presented from a general point of view. According to plans, the second volume will be devoted to the chemical constituents of essential oils. Succeeding volumes will describe individual essential oils including information on their botanical and geographical origins, methods of production, physical and chemical properties, and other significant data.

The first volume is divided into four chapters, each of which is written by an authority on the particular subjects covered. The origin and development of the essential oil industry is adequately covered in the first chapter by George Urdang.

In the second chapter the chemistry and origin of essential oils and the function of essential oils in plants are excellently presented by Dr. A. J. Haagen-Smit, Professor of Bio-organic Chemistry, in the California Institute of Technology.

Chapter three is devoted to the methods of distillation, enfleurage, maceration, and extraction methods used in the production of essential oils. This chapter was contributed by Dr. Ernest Guenther, Vice-President and Technical Director of Fritzsche Brothers, Inc., New York.

The fourth chapter by Mr. Edward E. Langenau, Director of Analytical Laboratories, Fritzsche Brothers, Inc., New York, includes detailed directions for the examination and analysis of essential oils, synthetics, and isolates.

The value of the book is further enhanced by an appendix which includes interesting information concerning the use and storage of essential oils and useful boiling-point data and conversion tables.

The text is well documented throughout. The binding and printing are of good quality and each page shows evidence of meticulous care in preparation. All who are concerned with essential oils will find Volume I of *The Essential Oils* interesting and useful and will look forward with keen anticipation to the appearance of the succeeding volumes.

A Catalog of Insecticides and Fungicides. Compiled by DONALD E. H. FREAR. *Vol. I, Chemical Insecticides.* Chronica Botanica Company, Waltham, Mass., Stechert-Hafner, Inc., New York, 1947. xii + 203 pp. 19.5 x 27 cm. Price, $6.50.

During the war, the spectacular success of DDT focused attention on the possibility of relatively simple chemical substances as highly efficient insecticides. Prompted by urgent need, Dr. Frear set up a project at Pennsylvania State College to catalog all known insecticides and fungicides to serve as a point of departure for future work in this field. Volume I of this catalog lists over 10,000 chemicals, plant species, and miscellaneous materials which have been tested for possible control of insects and plant diseases. Given under each substance is chemical name, synonyms, and complete formula, as well as the result of insect and fungus tests and one or more literature citations. Patent indexes, by countries of origin and by number, and author indexes will be helpful. Volume II, not yet released, will cover fungicides.